Kinetic and Physiological Responses of *Listeria monocytogenes* to Novel Non-Thermal Inactivation Treatments and their Application to Minimally Processed Seafood

by

Claudio da Rocha Bittencourt

Food Engineering (1st Class Honours)

Submitted in fulfilment of the requirements for the Degree of

Doctor of Philosophy

University of Tasmania, June 2009

This is to confirm that this thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Claudio da Rocha Bittencourt
June, 2009

This thesis is not to be made available for loan or copying for two years following the date this statement was signed. Following that time the thesis may be made available for loan and limited copying in accordance with the *Copyright Act 1968*.

Claudio da Rocha Bittencourt
June, 2009

Abstract

Listeria monocytogenes is a facultative anaerobic pathogen found in soil and water, on vegetation, food contact surfaces and in raw food materials including seafood. It grows at refrigeration temperatures and up to 14% sodium chloride making it almost impossible to control in fresh or minimally-processed seafood under aerobic or anaerobic storage conditions.

This thesis considers strategies to control growth of this bacterium on seafood using both established and novel non-thermal technologies and seeks to elucidate physiological mechanisms underlying one of the approaches, namely high pressure processing.

Chapter 1 reviews the microbiology of seafood including normal microbiota, spoilage processes, pathogen ecology and occurrence and non-thermal processes that are currently used or have the potential to be utilized by the seafood industry. It serves as reference material for the following chapters.

Chapter 2 describes an empirical assessment of several preservatives proposed as anti-listerial agents. In collaboration with a local Atlantic salmon smokehouse, three commercially available antimicrobial preparations were applied directly to salmon fillets prior to smoking with the intent to stop *Listeria monocytogenes* growth on vacuum packed cold smoked salmon (CSS). The challenge trial extended over 40 days at 4°C and 10°C. Microbial and sensorial analyses were conducted in parallel. Results showed that two of the three treatments evaluated presented listeriostactic activity. The remaining compound appeared not to penetrate in the salmon flesh and challenge the *Listeria monocytogenes* introduced to the inner flesh after slicing. From the sensorial point of view one of the successful *Listeria monocytogenes* growth inhibitors performed slightly better than the other but significantly better than the

untreated CSS, making it a good candidate to control the growth of this pathogen in this commodity.

A search for novel, cold-active, anti-listerial bacteriocins is the focus of Chapter 3. Specifically, 1600 Actinobacterial isolates from Antarctic or Sub-Antartcic regions were screened against five different *Listeria monocytogenes* strains for their capacity to produce cold active antimicrobials. Several promising isolates were identified and their active products partially characterized. The investigation demonstrated that Antarctic or Sub-Antarctic soils harbour potentially valuable antimicrobial producers with specific capacity to target single pathogen species and their potential to food safety and industry. Future bio-prospecting research for antimicrobials against pathogens of human concern should include species from other extreme environments as well.

Chapter 4 and 5 describe studies concerning physiological responses of *Listeria monocytogenes* to high pressure processing (HPP). Chapter 4 explores whether the cell membrane is an important mediator of the effects of HPP and reports studies of changes in the fatty acid composition of the membrane in response to HPP. The results suggested that under pressure, irrespective of the growth phase, *Listeria monocytogenes* tries to adapt by changing the abundance of iso branched-chain fatty acid of its cell membrane. This fatty acid adaptive response is different from that caused by cold, pH and heat stresses. In Chapter 5 microarray technology is used to assess changes in gene expression under the same experimental conditions showing that HPP seems to invoke a cell maintenance state but strongly suppresses genes associated with catabolism and virulence.

Chapter 6 synthesises the results of the work undertaken, attempts to determine the applicability of the novel non-thermal technologies (applied alone or as part of a multi

hurdle approach) to increase the safety of minimally processed seafood products against listeriosis and identifies future research needs.

Acknowledgments

It gives me a great pleasure to formally acknowledge the always friendly and supportive people at the School of Agricultural Science and Tasmanian Institute of Agricultural Research (TIAR), University of Tasmania.

I will start with the "more experienced" ones.

Professors Tom McMeekin, David Ratkowsky and June Olley. Thanks a lot for all of your support over the years helping me with statistical issues, experimental design and planning as well as execution and also for the informal but instructive chats. Your commitment to the students is truly exemplary and it will be remembered for many years to come. I may even miss Tom's jokes !! only if I could understand them...

To the staff and PhD candidates thanks for being around during the candidature as well as functions, parties, barbecues, soccer, camping, bushwalking, fishing and some many other shared moments. It was a funny time and is greatly missed in the current corporate days !

Special thanks goes to (not in any order) Sally, Lyndal, Jimmy, Julia, Panko, Bisso, Tim, Chen, Chris, Samuel, Ifa, Alistair, Jane, Heather, Mark, Kathleen, Esta, Liv, Alison, Shaun, Jimmy Kirkham, Rochelle, Danielle, Mandeep and so many others.

I thank the previous Head of School, Dr Peter Lane, for the opportunity given to me to be part of this exciting school. The previous post-graduate coordinator and current Head of School, Dr Phil Brown, is also thanked.

Financial support from Australian Postgraduate Awards (APA) and Top-up scholarship from the Australian Commonwealth Scientific and Research Organization (CSIRO) and Food Science Australia (FSA) is acknowledged and thanked.

A great deal of appreciation goes to my "adopted" co-supervisor Assoc. Prof. John Bowman especially towards the end of my thesis. Your promptness and always valuable comments were inspirational. Thanks a lot John.

To my only official supervisor, Assoc. Prof. Tom Ross, I thank you for your intellectual support, friendship in and out of the university, humbleness and fairness. I also thank you for the constructive criticisms. They are certainly welcome now.

Finally to the ones that mean the most to me. My parents, Pacifico and Neuza, and brother and sister, Ezio and Simone, thanks for being part of this achievement.

To my wife Catia, certainly the biggest role model in my life, I thank you for your kindness and support during this adventurous and sometimes treacherous journey. This is for me as much it is for you. Congratulations too !!!

Table of Contents

Introduction

The food industry is constantly evolving trying to deliver products that resemble their untreated or raw-like sensorial properties but with an enhanced level of food safety or longer shelf-life. Those parameters are not the interest of food safety regulators or quality assurance personnel only. Marketing and sales departments can make use of those points of differentiation among competitors to promote the products and secure valuable shelf space throughout supermarket or other retail chains.

Thermal processes are well established and although they provide consumer with safe ready-to-eat foodstuffs they fail to maintain some of the desirable intrinsic properties of the treated material making it non-relevant to a large number of products/applications. Colour variation, textural and flavour changes are some of the irreversible and undesired modifications caused by the heat process.

Some of the emerging and established non-thermal technologies to control *Listeria monocytogenes* in minimally processed seafood products as well as physiological adaptations of this bacterium to high pressure processing are discussed herein.

Chapter 1: Seafood Spoilage and Safety and Non-Thermal Technologies for the Seafood Industry

INTRODUCTION

The microorganisms present on fish originate from the natural microbiota of the raw material, which is dependent on the aquatic environment, and those organisms introduced in the course of processing and storage. The survival and growth of the various species present is determined by the properties of the seafood, its storage environment, properties of the microorganisms and the effects of processing. While most species of the microbiota have no effect on the food, some microorganisms cause spoilage of the product or may lead to foodborne illness. With today's growing interest in fresh and lightly preserved foods it is becoming increasingly important to understand the behaviour of microorganisms in fish and fish products to reduce losses due to spoilage and to assure the microbiological safety of seafood.

The purpose of this review is to:

(a) Describe the microbiota of fish and highlight the microorganisms that are of importance in fish spoilage and foodborne illness,

(b) Provide an overview of the emerging non-thermal processing options to increase the shelf life and microbiological safety of fish products.

MICROBIOLOGY OF FISH

The initial microbiota of fresh fish reflects the microbial population of their aquatic environment (Cahill 1990; Dalgaard 2006). Fish from temperate waters predominantly carry psychrotrophic, aerobic or facultative anaerobic Gram-negative bacteria such as *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Shewanella putrefaciens*, *Flavobacterium*, *Vibrio*, *Photobacterium*, *Aeromonas* and Enterobacteriaceae. *Vibrio*, *Photobacterium* and *S. putrefaciens* require sodium for growth and are typical inhabitants of marine waters, whereas *Aeromonas* spp. and Enterobacteriaceae are frequently isolated from freshwater fish. Gram-positive microorganisms isolated from fresh fish include *Micrococcus*, *Bacillus*, *Clostridium* and a variety of lactic acid bacteria (LAB)[1]. The microbiota of tropical fish often carries a slightly higher load of Gram-positive and enteric bacteria. The initial microbiota of fresh fish also varies between cultured and wild fish because the former tend to be closer to land and human activities. For example, *Listeria monocytogenes*, a bacterium pathogenic to humans, is not present in open waters but is found in decaying plant material and, therefore, can contaminate fish cultured in areas with run-off water from land. Further, fertilization by animal excreta may result in contamination with *Salmonella*, *Escherichia coli* and enteric viruses.

Microorganisms are found on the outer surfaces (skin and gills) and in the intestines of live and newly caught fish. The total numbers of culturable microorganisms vary enormously and in general are 10^3-10^5 colony forming units (CFU)/cm^2 on skin, 10^3-

[1] Lactic acid bacteria encompass a wide range of Gram-positive bacterial species that, by definition, produce lactic acid. They typically grow well under vacuum-packaging and are relatively tolerant to salt and acid.

10^7 CFU/g on gills and 10^7- $>10^8$ CFU/g in the intestinal content, the latter being related to the fish's intake of food (reviewed by Dalgaard 2006). At the time of slaughter fish muscle is sterile but becomes contaminated by surface and intestinal bacteria (indigenous bacteria) and from equipment and humans during handling and processing (non-indigenous bacteria). The ability of the contaminating microorganisms to survive or even grow on fish is dependent on intrinsic and extrinsic properties of the fish (*see* Table 1) and the microbe's ability to tolerate those conditions.

Table 1 Intrinsic and extrinsic factors that affect microbiological survival and growth

Intrinsic factors	Extrinsic factors
Product structure	Storage temperature
pH and acidity	Light exposure
Composition and formulation	Packaging
Water activity and moisture content	Relative humidity
Oxygen availability and redox potential	Gaseous atmosphere
	Hygiene of processing and handling

The composition of fresh fish makes it highly favourable to microbial growth (reviewed by Gram 2006). Typically characterized by neutral pH, fresh fish are rich in water, protein and, in some species, lipids and fish muscle contains free amino acids and nucleotides. Many fish, particularly those from seawater environments, contain trimethylamine oxide (TMAO), a compound involved in osmoregulation that

can also stimulate microbial growth and activity. Some species of bacteria are able to reduce TMAO to trimethylamine (TMA) and this anaerobic respiration facilitates their growth under oxygen limiting conditions (e.g. in vacuum packed, "VP", or modified atmosphere packaged, "MAP", products). TMA contributes to the typical ammonia-like and fishy off-odours in spoiled seafood (*see* Section "*Microbial Spoilage*"). Furthermore, while fish products are most often chilled, the initial microbiota of fish from temperate waters includes psychrotolerant species that are able to grow at temperatures above -2°C.

The growth and survival of microorganisms on fish is inhibited by some processes involved in seafood production and by storage at low temperatures. For example, the production of cold smoked salmon (CSS) includes salting, drying and cold-smoking that, in combination with the raw material used, imparts the product's flavour but also contributes to the microbiological safety and shelf-life of the final product. The processes of salting and drying reduce the amount of water available to microorganisms in the salmon (i.e. they lower the water activity, a_w, of the product), which reduces microbial survival and growth. Smoking adds smoke compounds to the fish muscle that have antimicrobial properties, possibly due to the combined action of formaldehyde, phenolic substances and acids in smoke (Suňen 1998, Thurette *et al* 1998, Niedziela *et al* 1998, Faith *et al* 1992, Messina *et al* 1988). Lowered a_w and the antimicrobial activity of smoke components are the primary preservation pressures in CSS although other factors can contribute (Leroi *et al* 2000; Leroi and Joffraud 2000). For example, CSS is typically VP which inhibits the growth of strict aerobes. Some products are MAP which can select against particular organisms if they are sensitive to the gaseous mixture used. The addition of nitrite, which imparts a specific flavour and colour to food products, inhibits microbial

growth including inhibition of germination of spores including *C. botulinum* type E and nonproteolytic types B and F, however, because of long-term health concerns associated with dietary nitrite, the level of added nitrite is strictly regulated. In the US, 200 ppm sodium nitrite ($NaNO_2$) is the maximum level allowed and the addition of nitrite is not allowed in seafoods in Europe (European Parliament and Council 1995; FDA/ORA 1996). In Australia, the use and amount of sodium nitrite permitted in fish and fish products is not specified, although meat and meat products, such as cured meat, dried meat and slow dried meat, may contain up to 125 ppm (FSANZ 2006).

The storage of CSS at temperatures < 5°C is critical to reduce spoilage and disease. Importantly, the manufacture of CSS does not involve any high temperature treatment and as a ready-to-eat (RTE) product, CSS is generally not cooked prior to consumption. Table 2 shows the characteristics of CSS.

Table 2 Characteristics typical of cold smoked salmon products (compiled from published literature)

Characteristic	***Range***
Water activity (or water phase salt, WPS*)	0.950-0.983 (3-8%)
Phenols (from smoke)	3-13 ppm
pH	5.9-6.3
Lactate	4000-15000 ppm
Nitrite salts	0-220 ppm

*WPS = (% NaCl x 100) / (% NaCl + % H_2O) (Hilderbrand, 1991)

The majority of microbial species found on fresh fish have no recognized importance in seafood, however, spoilage and human disease are caused by specific groups of microorganisms that are able to survive and/or grow on fish following processing and storage.

FISH SPOILAGE

The term 'spoilage' refers to changes in a food product which renders it unacceptable for the consumer from a sensory perspective (Dalgaard 2006). In fish, spoilage is typically characterised by gas production, formation of slime on the fish surface and gills, discolouration, textural changes and the development of off-flavours and off-odours. Such spoilage causes significant product losses and is caused predominantly by microbial growth and metabolism, although chemical reactions and the fish's own enzymatic reactions make some contribution.

Microbial Spoilage

On newly processed fresh, or lightly preserved, fish microorganisms that will later cause spoilage are usually not the dominant species present but exist initially in very low numbers. During storage, and at particular conditions of intrinsic and extrinsic factors, these species grow faster than the remaining microbiota and produce metabolites that are responsible for the sensory rejection of the product. Constituting a single or multiple species, the microorganisms responsible for spoilage of a product are termed the specific spoilage organism (SSO). When products are spoiled, the level of SSO is typically 10^7-10^8 CFU/g (Huss, 1993; Dalgaard 2006).

Identification of an SSO for a particular fish product relies on a comparison of the spoilage characteristics of the product with the activity of microbes contaminating

the fish. The microbiota present when the product is spoiled constitutes a number of bacterial species (i.e. the 'spoilage microbiota'), some of which are not important for spoilage. Each bacterium is isolated and tested for its 'spoilage potential', i.e. its ability to produce sensory changes consistent with the naturally spoiled product and, where spoilage potential exists, the capacity for that microorganism to grow in the fish to a level sufficient to cause sensory rejection is tested. Where growth is adequate, the microorganism is said to have 'spoilage activity' and verification in the food product is required to determine if it is the SSO (Gram 2006).

The SSO responsible for the spoilage of fish depends on the initial microbiota and the storage of the product and typical species are summarised in Table 3. During storage of fish in ice, psychrotrophic pseudomonads and shewanellae become dominant (Gennari and Tomaselli 1988; Tryfinopoulou *et al* 2002; Gram 2006). *Pseudomonas* spp. spoil freshwater fish when counts reach 10^8-10^9 CFU/g which is indicated by sweet, sulphydryl, rotten off-odours due to the production of esters and sulphydryl compounds (Gram and Huss 2000). *Pseudomonas* spp. are unable to reduce TMAO and their growth is considerably reduced under oxygen limited conditions (e.g. in VP-CSS). In contrast to freshwater fish, the spoilage of marine fish stored in ice is characterised as putrid, rotten and 'fishy' due to formation of hydrogen sulfide (H_2S) and TMA from the anaerobic respiration of TMAO by psychrotrophic *Shewanella* spp (Gram 2006). Other SSO in freshly chilled and aerobically stored fish include *Photobacterium phosphoreum*, which has been shown to be responsible for TMA formation in cod fillets and squid (Paarup *et al* 2002; Larsen *et al* 2003) and dominates the spoilage biota in saithe and plaice (Dalgaard *et al* 1997). *Psychrobacter immobilis* can dominate the spoilage microbiota in both marine and freshwater fish and increases the rancid spoilage of sardines (Gennari *et al* 1999). When fish are left

at ambient temperature, spoilage occurs very quickly (12-48 hours) and is due to the microbial growth and activity of *Vibrio* and *Aeromonas* species (Huss 1993; Gram 2006).

Table 3 Examples of the specific spoilage organism(s) in seafood products (Source: Dalgaard, 2000)

Product	***Typical specific spoilage organism***
Fresh, chilled fish stored in air	*Shewanella putrefaciens*[1], *Pseudomonas* spp.[2]
Fresh, chilled fish vacuum- or modified atmosphere-packed	*Photobacterium phosphoreum*[1], Lactic acid bacteria[2], *Brochothrix thermosphacta*[2]
Fresh fish stored at >10-15°C in air	Vibrionaceae, Enterobacteriaceae
Cooked and brined modified atmosphere packaged shrimps and possibly other packed, lightly preserved seafood stored at 15-25°C	*Enterococcus faecalis*

[1] Typical of marine, temperate-water fish; [2] Typical of freshwater or tropical water fish

MAP with carbon dioxide (CO_2) of fish from marine waters causes selection of CO_2 resistant, psychrotolerant *P. phosphoreum* (Dalgaard *et al* 1993). *P. phosphoreum* is inactivated by freezing and is absent in thawed fish products (Guldager *et al* 1998; Emborg *et al* 2002). Fish from freshwater probably do not harbour this bacterium and in these products LAB tend to become the dominant spoilage organism present. *Aeromonas* spp. have been found in chilled MAP seafood from tropical regions and may be the SSO (reviewed by Dalgaard 2006). Further,

where processing techniques used are novel, or unusual amongst seafood production, changes in the spoilage microbiota may occur (reviewed by Dalgaard 2006). For example, low dose irradiation of chilled seafood leads to domination by *Moraxella* spp. which is probably responsible for spoilage. In *sous vide* cooked and chilled cod fillets, *Clostridium sardiniense* produces volatile sulphur compounds and leads to product rejection.

Studies relating specifically to lightly preserved fish products, including VP-CSS, have determined the SSO to be Gram positive bacteria such as LAB, *Brochotrix thermosphacta* and, to a lesser extent, the Gram negative Enterobacteriaceae and Vibrionaceae (Basby *et al* 1998; Leroi *et al* 1998; Jørgensen *et al* 2000). These microorganisms are better able to grow in the fish after the addition of small amounts of NaCl and acid. However, if the product is temperature abused or otherwise inadequately preserved (e.g. too little NaCl or acid added during processing) *S. putrefaciens* might also contribute to product spoilage. *P. phosphoreum* is a SSO in MAP-CSS containing CO_2. Variation in product characteristics, including the initial microbiota, NaCl, pH, smoke components, chemical preservatives and packaging causes variation in the spoilage patterns observed in chilled and lightly preserved seafood.

The characteristics of spoilage that occur in VP-CSS can provide an indication of the SSO responsible. For example, putrid, sickly, sulphurous off-odours and off-flavours tend to be caused by Gram negative rods (Enterobacteriaceae and Vibrionaceae) and occasionally LAB, whereas sourness and acid off-flavours, or complete loss of aroma, are due to LAB (Truelstrup Hansen 1995; Leroi *et al* 1998; Huss 1993). Interactions between species within the spoilage microbiota can also influence the spoilage of the product. For example, in CSS the production of

putrescine by some Enterobacteriaceae is increased in the presence of LAB (Jørgensen *et al* 2000). Microorganisms also interact in terms of competition for substrates and the production of metabolites that may inhibit the growth of other species. The dominating microbiota typically inhibit the growth of other microorganisms and, therefore, it is important to remember that any changes in processing of fish that alters the dominant species within the microbiota may affect both the spoilage and safety characteristics of the product.

Spoilage of fish products can occur very early in the storage of the final product but the actual time can be very variable. Changes to current manufacturing regimes might better inhibit the growth of the SSO and, therefore, offer the potential to increase the time to spoilage of the product. This will be dealt with in more detail in the Section "*Non-Thermal Processing Alternatives*", below. It is important to note that while modifications to the processing of fish could inhibit the growth of a known SSO, such changes might also allow other spoilage organisms, or even pathogenic species, to grow to higher levels due to a reduction in competition for resources and other changes in the environment. These events could pose considerable risk within the final product and must be considered when altering processing techniques.

The use of mathematical models to predict the growth rate and metabolism of the SSOs as well as pathogens such as *L. monocytogenes* and *C. botulinum* are an area of the microbiology denominated Predictive Microbiology (McMeekin *et al.*, 1993; Mejlholm Dalgaard, P. (2007). The models are based on real observations in experiments carried out on different broths and/or food matrixes, temperatures, pH, atmospheres and NaCl contents.

The qualitative and quantitative composition variation of the indigenous microflora in any given product offers a great challenge to predictive microbiologists

specially when the models are designed to fit an environment but are used in another one with completely different bacteria spp or processing parameters. For example, the microbial load, including the SSOs, on a northern hemisphere fish may be different than the one on the southern hemisphere. Consequently, models need to be specific to the pathogen or SSO or relevance to the product. Another example is the cold smoking process where hard and soft wood would produce different levels of antimicrobial compounds affecting the safety and shelf-life of the smoked product. In both cases, a more specifically catered model would produce more reliable predictive results.

There are several predictive models that can be freely downloaded from internet. A list of a few predictive microbiology application software, in addition to Seafood Spoilage and Safety Predictor (SSSP, http://sssp.dtuaqua.dk/), can be found at the following link:

http://sssp.dtuaqua.dk/HTML_Pages/Help/English/Other-software/Other-software.htm

It can be expected that the accuracy and spread use of predictive models will increase with time, and they are already beginning to play an important role in the management of microbial food safety and quality.

Chemical Spoilage

The major chemical reaction in fish that results in spoilage is the oxidation of polyunsaturated fatty acids that produces hydroperoxides. Hydroperoxides can cause brown and yellow discolouration of fish tissue and are broken down to aldehydes and ketones, which impart a strong, rancid flavour in fish. Heat, light (particularly ultraviolet light, UV) and several organic and inorganic substances (e.g. copper ions, Cu^{2+}, and iron ions, Fe^{2+}) initiate or accelerate the oxidation of fatty acids.

Autolytic Spoilage

Autolysis means "self-digestion" and this kind of enzymatic spoilage is the first to occur and affect the quality (or the loss of it) of fresh fish (reviewed by Huss 1995). Table 4 lists the most important enzymes in chilled fish spoilage, their effects and methods to prevent or inhibit them. Fortunately, autolytic spoilage is marginal in chilled fish products because reactions are slowed by low temperatures except in some ungutted fish where the high levels of gut enzymes cause off-odours and discolouration.

Table 4 Summary of autolytic changes in chilled fish (Modified from Huss 1995)

Enzyme(s)	*Substrate*	*Changes Encountered*	*Prevention/Inhibition*
Glycolytic enzymes	Glycogen	Production of lactic acid; reduction in pH of flesh; loss of water binding capacity of muscle; high temperature rigor may result in 'gaping'	Fish should be allowed to pass through rigor close to 0°C; avoid pre-rigor stress
Nucleotide-degrading enzymes	ATP, ADP, AMP, IMP	Loss of fresh fish flavour, gradual production of bitterness	As above; reduce rough handling or crushing of product
Cathepsins	Proteins, peptides	Softening of tissue making processing difficult or impossible	Minimise rough handling during storage and discharge
Chymotrypsin,	Proteins,	Autolysis of visceral cavity	Problem increased

trypsin, carboxy-peptidases	peptides	in pelagics (belly- bursting)	with freezing/thawing or long- term chill storage
Calpain	Myofibrillar proteins	Softening, molt-induced softening in crustaceans	Removal of calcium
Collagenases	Connective tissue	'Gaping' of fillets; softening	Related to time and temperature of chilled storage
TMAO demethylase	TMAO	Formaldehyde-induced toughening of frozen gadoid fish	Store fish at ≤ -30°C; reduce physical abuse and freezing/thawing

Importantly, autolytic spoilage is a significant concern in frozen fish. Freezing of fish inhibits microbial activity including the reduction of TMAO to TMA by specific microorganisms and instead TMAO is converted by autolytic enzymes to dimethylamine and formaldehyde. Formaldehyde causes denaturation of the fish tissue, changes in texture and loss of water binding capacity.

MICROBIOLOGICAL SAFETY OF FISH

Consumption of fish can result in disease in humans due to the growth and/or survival of pathogenic microbes or the production of poisonous compounds by microbial activities. Microbiological hazards in fish involve a number of indigenous microorganisms (i.e. those present on the fish from the aquatic environment) including *Clostridium botulinum*, *L. monocytogenes*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Vibrio vulnificus*, *Anisakis* spp., and histidine decarboxylase positive

bacteria, such as *Photobacterium phosphoreum*, *Photobacterium damsel*, *Morganella psychrotolerans* and *Morganella morganii*. Non-indigenous pathogenic bacteria that are important contaminants of fish include *L. monocytogenes*, *Salmonella*, *Shigella*, pathogenic strains of *E. coli* and *Staphylococcus aureus*. Importantly, non-indigenous bacteria can be prevented from contaminating CSS during production if sufficient attention is given to good manufacturing practices (GMPs), sanitation standard operating procedures and good hygienic practices GHPs[2].

The more significant health concerns associated with the consumption of fish products are described below.

Histamine Fish Poisoning

Histamine fish poisoning (HFP) is a mild disease that occurs quickly following consumption of fish containing >500 ppm histamine. Symptoms include rash, inflammation, nausea, vomiting, diarrhoea, headaches and hypotension. Microorganisms in fish that produce the enzyme histidine decarboxylase are responsible for the formation of histamine from histidine. Bacterial species known to be involved include some species of Enterobacteriaceae (e.g. *Enterobacter aerogenes*, *Enterobacter cloacae*, *M. morganii*, *Proteus vulgaris* and *Raoultella planticola*), *Vibrionaceae*, and LAB (reviewed by Dalgaard 2006). Importantly, HFP is limited to seafood products where histamine production occurs at high levels (i.e. where there is a high content of free histidine, histidine decarboxylase producing microbes are able to grow to high numbers and where the histidine decarboxylase has high activity). Therefore, HFP most often occurs in anchovy, bluefish, bonito, herring, mackerel,

[2] For relevant and extensive information on GMPs and GHPs see USFDA (2001) and Huss (1993).

dolphin fishes, marlin, garfish, swordfish, tuna and yellowtail (Lehane and Olley 2000). Histamine was believed not to be produced when fish was stored below 5°C but that it could reach high levels when product was stored above 7°C. Once formed, histamine is heat stabile and will not be destroyed by traditional cooking methods (Lehane and Olley 2000). More recently Emborg *et al* (2005) have demonstrated histamine formation by psychrotolerant bacteria on tuna at 2°C.

A number of governing bodies have introduced regulations relating to the maximum levels of histamine in fish (e.g. the US Food and Drug Administration, USFDA, requires < 50 ppm[3]). To maintain levels below such limits, susceptible fish products should be chilled to <5°C and storage time should be limited. Freezing can inactivate the histamine-producing bacteria but histidine decarboxylase will remain active at freezing temperatures and so this method is limited in its effectiveness.

Vibrio Species

Commonly associated with the consumption of raw, temperature-abused or undercooked seafood, including sashimi and sushi, *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* are the most important seafood-borne human pathogens of this genus. Outbreaks of disease are common in the US, Japan and other areas of Asia but incidents are significantly lower in Europe despite a recent increase in the consumption of raw fish products. In Australia, prawns and oysters were the most probable sources of four *V. parahaemolyticus* outbreaks between 1980 and 1995 and *V. vulnificus* was implicated in four cases (including two deaths) in New South Wales in 1988 (Kraa 1995). The incidence of *V. cholerae* outbreaks in Australia appears

[3] Note that this limit was introduced as an indicator of product quality (or the occurrence of temperature abuse) rather than indicating a level considered unsafe for consumption.

much lower, with an average of less than one case per annum (Kraa 1995; Anonymous 1999). The viability of pathogenic *Vibrio* spp. in fish is reduced by storage at 0-5°C, freezing, normal cooking procedures and high-pressure processing (Gram and Huss 2000; Cook 2003).

Although the large majority of *V. parahaemolyticus* in seafood are non-pathogenic, pathogenic variants cause a gastrointestinal disease in humans when approximately 10^6 cells are consumed (Kaysner 2000; Cook *et al* 2002). Therefore, to cause disease other than from filter-feeding shellfish, *V. parahaemolyticus* must be able to grow during storage of the seafood product. Oysters in seawater above 15°C are the primary vehicle for *V. vulnificus* disease in humans (Lorca *et al* 2001). *V. vulnificus* causes septicaemia that is characterised by fever, chills, nausea and hypotension and can be fatal in up to 50% of cases. Infection is rare, however, and limited to a small proportion of consumers with pre-existing illnesses that predispose them to infection. *V. cholerae* is the causative agent of cholera, a gastrointestinal disease that causes severe watery diarrhoea that result in dehydration, and in some cases, death. Epidemics of cholera are caused by the two serotypes O1 and O139 that produce cholera toxin and are present on fish due to contamination of water with sewage. Therefore, good sanitation practices can prevent the incidence of cholera caused by *V. cholerae* O1 or O139. *V. cholerae* non-O1/non-O139 cause sporadic cases of milder disease and occur naturally in estuarine waters.

Salmonella

Salmonella is a member of the Enterobacteriaceae. It is a gram negative, non-sporing rod that is widely distributed in nature. It is responsible for the potentially fatal disease salmonellosis, which is characterised by vomiting, nausea, abdominal pain

and diarrhoea and typically has an infective dose of approximately 10^6 cells, although the numbers may be much lower in some circumstances. In relation to its presence in fish, *Salmonella* is rare in temperate waters but occurs in fish from tropical estuaries and coastal waters. Importantly, humans and vertebrate animals are the primary reservoir of *Salmonella* and, therefore, contamination of fish can occur during and after processing where poor sanitation occurs. Relative to other types of food, seafood has caused few cases of salmonellosis but the presence of *Salmonella* in fish is grounds for product rejection on importation to Europe or the US (D'Aoust 2000). *Salmonella* grow at > ~5 C, pH > 3.8 and with < 6% NaCl but are inactivated by normal cooking and smoking at high temperatures. Post-processing *Salmonella* contamination of raw ready-to-eat seafood is a risk to consumers.Good manufacturing practices (GMPs) must be implemented to minimize this hazard from occurring..

Clostridium botulinum

C. botulinum is a Gram-positive, anaerobic bacterium capable of forming spores which allow them to survive in a dormant state until exposed to conditions that can support their growth. *C. botulinum* produce 'botulin', a toxin that causes severe neurological dysfunction (i.e. weakness, total paralysis and respiratory failure) that can be fatal in humans. Mortality has been reported to be as high as 75% (Lund and Peck 2000; Gram 2001). While human botulism is relatively rare (for example in Australia there were five reported cases of botulism between 1942 and 1983, of which four were linked to vegetables and one to canned tuna (Hauschild 1993)), its implications are very serious and, therefore, it is a significant concern to the food industry. Where fishborne outbreaks of botulism have been reported, lightly preserved products are most frequently implicated, as shown in Table 5.

Table 5 Type of fish products causing botulism (period 1950-1980; 165 cases). Source: USFDA 2001

Fish product	*Process used*	*Number of outbreaks*
Lightly preserved	Smoked	10
	Fermented	113
Semi-preserved	Salted	9
	Pickled	8
Fully preserved	Canned	5
Unknown		20

There are seven types of botulism toxin designated A through G; only types A, B, E and F are pathogenic to humans. Proteolytic strains of *C. botulinum* classified as Group I produce toxins of type A, B and F and the non-proteolytic strains, classified as Group II, produce toxins of type E, and some B and F (Huss *et al* 2003). *C. botulinum* group I produce highly heat resistant spores, are tolerant to salt, are mesophilic (i.e. optimum temperature for growth is 30-45°C and minimum temperature for growth of approximately 10°C) and are found throughout the environment. In contrast, group II produce relatively heat sensitive spores and are salt sensitive, psychrotolerant (i.e. optimum temperature for growth is up to 20°C), and are most commonly found in the aquatic environment.

Aquatic sediments collected throughout Europe and North America have shown that *C. botulinum* non-proteolytic type E is the most frequent *C. botulinum* found in those environment. Its prevalence in sediment is close to 100%, with the

exception of the British Isles (Huss 1981; Dodds 1993). As a consequence, it is possible that 0.4 to 100% of freshly caught fish could be contaminated with *C. botulinum* type E spores (Huss 1981; Lund and Peck 2000). In Australia, *C. botulinum* type A, B and C have been isolated from soils and waterways (Ross and Sanderson 2000), however, an attempt to isolate *C. botulinum* type E from 528 samples from soil, marine mud, fish intestines and potato washings from Tasmania, New South Wales and Queensland was unsuccessful with no positive results (Christian 1971). While the occurrence of *C. botulinum* in the environment can be high, the incidence of this species in processed fish, such as CSS and smoked eel, is significantly lower (1.5 to 19.6%) as suggested by Huss (1981) and Lund and Peck (2000). The USFDA (2001) has also released information regarding the prevalence of *C. botulinum* type E in fish (see Table 6).

The human pathogenic toxins produced by *C. botulinum* have a very low lethal dose and are resistant to acid and NaCl. They are, however, sensitive to heat and alkaline pH and are inactivated when held at 79°C for 20 minutes or at 85°C for 5 minutes (Lund and Peck 2000). Therefore, cooking fish is very important for ensuring safety. Where fish products are minimally processed and do not include a heating step, the risk of botulism is increased. Fresh seafood stored aerobically at below ~10°C is generally spoiled before *C. botulinum* toxins can be detected but toxin formation increases with temperature abuse. Oxygen tends to delay toxin formation by *C. botulinum* but aerobic storage or MAP with oxygen cannot

Table 6 Prevalence of *C. botulinum* type E in fish (Source: USFDA 2001)

Fish type	*Sample size, g*	*% positive*	*MPN[2] / kg*
Whitefish	10	12 (also C)	14
Flounder, vacuum-packed, frozen	1.5	10	70
Rockfish, dressed	10	100	2400
Cod, whiting, flounder	?	0.40	-
Salmon, smoked	20	2 (type B)	< 1
Carp, salted	2	63	490
Cod, haddock a.o.	10 intestines	4.5	40-100
Trout, farmed	whole fish	5-100	340-5300
Trout, herring	80-234	0	-
Herring	whole fish	45-65	5 - 60
Rainbow trout, cold-smoked	100-200	3[1]	40-290
Rainbow trout, fresh	100-200	15-20[1]	30-1900

[1] Detection of type E toxin gene by polymerase chain reaction; [2] MPN, most probable number

totally prevent it (Dufresne *et al* 2000; Gram and Huss 2000). *C. botulinum* group II are able to grow and produce toxin at approximately 3°C and in foods with less than 5% NaCl and pH > 5.0, although a combination of low temperature, high NaCl and/or low pH may prevent its growth (reviewed by Dalgaard 2006). For example, the growth of *C. botulinum* type E in lightly preserved fish products is generally controlled by proper refrigeration (<3.3°C, *see* Table 7) and increased salt content (i.e. WPS of ≥3.5% for VP-CSS or ≥2.5% for air packaged CSS). As indicated in Table 7, the control of *C. botulinum* group II requires more stringent temperature control than that of *C. botulinum* group I because the former are able to grow at low temperatures.

Table 7 Time/storage temperature guidance for controlling germination, growth and toxin formation of *C. botulinum* in seafood (Source: USFDA 2001)

***C. botulinum* group**	**Temperature (°C)**	**Maximum cumulative exposure time**
I	10-21	11 hours
	>21	2 hours
II	3.3-5	7 days
	6-10	>2 days
	11-21	11 hours
	>21	6 hours

Listeria monocytogenes

L. monocytogenes, the only human pathogenic strain of the *Listeria* genus, is a non-spore-forming Gram-positive, psychrotrophic, aerobic to facultative anaerobic bacterium. It is ubiquitous in nature and has been isolated from soil, marine sediments, water and vegetation (Price and Tom 2003). Although healthy individuals can carry *L. monocytogenes* in their intestinal tract without being affected by it, this bacterium is an opportunistic pathogen that causes severe infections in susceptible hosts (i.e. the young, elderly, pregnant and individuals with immune deficiencies). In pregnant women, *L. monocytogenes* can cause abortion and neonatal sepsis and in other susceptible people can cause severe infections such as septicaemia and meningoencephalitis. McLauchlin (1997) has reported the mortality rate of listeriosis infections to be as high as 30% and, in the US alone, *L. monocytogenes* is estimated to

cause approximately 2500 cases of human illness, including 500 deaths, annually (Kanuganti *et al*, 2002) although only about 50% of these cases are identified and reported.

The incidence of *L. monocytogenes* in CSS varies from < 1% (Valenti et al. 1991; Dillon et al. 1994; Garland 1995) to 80% after processing (Farber 1991; Hudson et al. 1992; Jørgensen and Huss 1998). Rørvik *et al* (1995) and Eklund *et al* (1995) have shown that the natural level of *L. monocytogenes* on freshly produced CSS is, in most cases, less than 10 CFU/g and, more rarely, between 10 and 100 CFU/g but this can increase upon storage even at cold temperatures (Jørgensen and Huss 1998). A risk assessment by the USFDA (2001) in collaboration with the US Department of Agriculture concluded that 15% of all smoked fish is contaminated with *L. monocytogenes.* A large body of evidence shows that *L. monocytogenes* may be present in raw salmon or any fish from similar environments in low numbers because the seawater from estuarine environments and coastal areas are constantly exposed to potential contamination with *L. monocytogenes* (USFDA 2001).

Molecular typing studies carried out by Rørvik *et al* (1995) and Autio *et al* (1999) have indicated that indigenous *L. monocytogenes* is not the primary source of this bacterium in raw or minimally processed salmon and trout. Instead, it appears that indigenous *L. monocytogenes* are replaced during production by other types of *L. monocytogenes* that contaminate the raw material during processing. The recontamination is likely to come from niches of *L. monocytogenes* (i.e. injectors, biofilms in tables, belts, slicers, cutting boards, etc.) that survive the sanitation step. Good sanitation practices in the processing environment are therefore critical for minimising the risk of *L. monocytogenes* infection in humans.

L. monocytogenes can grow with or without oxygen and over a wide range of environmental conditions, as shown in Table 8. *L. monocytogenes* is inactivated by normal cooking procedures and inhibited by the high concentrations of acetic acid (~2.5%) used in some semi-preserved seafood such as marinated herring. While the presence of low numbers of *L. monocytogenes* on seafood does not, in itself, represent a serious health threat, *L. monocytogenes* is capable of growing in many products with extended shelf-lives (Barakat and Harris 1999; Rørvik *et al* 1991) and, thus, presents a serious risk in foods that do not require heat treatment prior to consumption, including CSS. In such seafood products, the prevention of listeriosis is by good hygiene practices (GHPs) within the processing environment to reduce contamination of fish with *L. monocytogenes*, control of product characteristics to inhibit the growth of *L. monocytogenes* (e.g. MAP with CO_2), and limiting the shelf-life of the fish product stored at specific temperatures.

Table 8 Growth ranges for *L. monocytogenes* in response to factors relevant to fish products (Source: USFDA 2001)

Parameter	***Range for growth***
pH	4.4-9.4
Temperature	-0.4- 45 °C
Water activity	>0.92

The processing of a number of ready-to-eat (RTE) fish products does not include critical control points for *L. monocytogenes* and this is of significant concern in the production of microbiologically safe seafood. A number of new or modified non-thermal processes offer the potential to minimise the risk of *L. monocytogenes*

infection due to the consumption of fish products, as detailed in the Section "*Non-Thermal Processing Alternatives*".

PRODUCT SHELF-LIFE

The shelf-life of a product can be defined as the period for which it will retain an acceptable level of eating quality from both a safety and a sensory perspective (Singh and Cadwallader 2003). Developments to extend shelf life in fish products are numerous and some are reviewed in the Section "*Non-Thermal Processing Alternatives*". Indices of freshness or spoilage of seafood are based on physical, chemical and microbiological changes. Most of the physical changes are caused by improper handling/storage of the foodstuff (i.e. freezer burn, moisture migration, damage to packaging, and texture and flavour changes due to thawing and refreezing) and can be prevented with good manufacturing practices (GMPs). The most important chemical changes are associated with enzymatic reactions, oxidative reactions (particularly lipid oxidation) and non-enzymatic browning. A number of techniques, such as high performance liquid chromatography, gas chromatography and artificial electronic noses, have been used to quantify the volatile and non-volatile compounds produced under certain environmental conditions (i.e. pH, temperature, a_w, modified atmosphere). Chemical markers such as TMA, which causes off-flavours, and total volatile nitrogen are often used as quality indicators to characterise spoilage in seafood.

Microbiological shelf-life of some seafoods can be assessed by the total viable count, or aerobic plate count (APC), technique. This technique estimates the total numbers of bacteria per gram in a given product and is commonly used to verify the shelf-life of a product, giving an overall bacterial count instead of a specific count for

a single bacterial species. Table 9 shows earlier recommended APC limits for fisheries products set by the International Commission on Microbiological Specifications for Foods but which are still embedded in legislation in many countries. APCs below "m" are considered to be of good quality. Numbers between "m" and "M" are considered of marginally acceptable quality, but can be accepted as long as the number of samples are not higher than "c". Counts exceeding "M" are of unacceptable quality. In summary, if five samples are APC tested, all must be less than 10^7 CFU/g at least 2 must be less than 5 x 10^5 CFU/g so the product can be accepted.

Table 9 Recommended microbiological limits for fresh and frozen fish and cold-smoked fish (ICMSF 1986)

n	*c*	*m*	*M*
5	3	5 x10^5 CFU/g	10^7 CFU/g

n = number of representative sample units,

c = maximum number of acceptable sample units with counts between "m" and "M",

m = maximum recommended counts for good quality products,

M = maximum recommended counts for marginally acceptable quality products

It is now generally accepted that there is a poor correlation between remaining shelf-life, as determined by sensory methods, and APCs. However, much closer correlation has been observed between remaining shelf-life and the concentration of specific microbes known to cause spoilage, i.e. the SSO. The numbers of H_2S-producing bacteria in chilled and aerobically stored fish has been correlated with

product spoilage, as have the levels of *P. phosphoreum* in cold-stored MAP cod fillets (reviewed by Dalgaard 2006). However, to use such assessments, knowledge of the physiology of the dominant spoilage organisms in a given product is essential to select appropriate methods for enumeration, e.g. which incubation temperature should be used to truly quantify the appropriate SSO. For many fish and fish products, a plate incubation temperature of 25°C produces a significantly higher number of bacteria than incubation at 35°C (Nickelson and Finne 1992). This discrepancy can also be noted if inadequate media, lacking in nutrients required by the SSO, are used.

The accurate prediction of product shelf life has become increasingly important to reduce product losses. Predictive microbiology has provided a means to estimate the effect of product characteristics and storage conditions on the growth and survival of the SSO or some pathogenic species. For example, kinetic models have been developed to predict the growth of *P. phosphoreum* and *S. putrefaciens* as a function of temperature and CO_2 in MAP seafood (reviewed by Dalgaard 2002). The development of predictive models relating to other microorganisms is likely to progress more quickly in the near future due to improved access to growth and survival data collected in on-line databases like ComBase (http://www.combase.cc/).

NON-THERMAL PROCESSING ALTERNATIVES

Developments in classical and emerging technologies to extend shelf life of seafood are numerous. In many cases, these procedures are aimed at inhibiting the growth or survival of a particular SSO or pathogenic bacterium that is of concern in a specific product. For example, laboratory studies determined a specific combination of NaCl, sorbate and smoke components to inhibit *Aeromonas*, an SSO in Nile perch stored at ambient temperature, and, thereby, developed a lightly preserved product with marked

increase in shelf life (Gram 1991). Other examples include the inhibition of *P. phosphoreum* in chilled MAP cod fillet by oregano essential oil or by freezing, which is lethal to *P. phosphoreum* (Emborg *et al* 2002; Mejlholm and Dalgaard 2002).

It is important that any changes to a standard processing protocol are investigated for their effect on the growth and survival of pathogenic or spoilage bacteria of current concern as well as other microorganisms that may be better able to grow and survive. For example, if salt content is increased to 5.5% in CSS to reduce spoilage LAB, there is potential for pathogens, including *L. monocytogenes*, to grow to dangerous levels because the inhibitory effect of competitive microbiota is reduced. Inhibition of the growth and/or activity of spoilage microorganisms to increase product shelf life also allows more time for pathogenic species to grow in the food. Jay (1996) and Jay (1997) have suggested that the increasing number of foodborne disease outbreaks in the US can be partly attributed to the reduction of competitive background microbiota by mild preservation steps (e.g. drying, salting, packaging, use of antimicrobials, etc.), which allows pathogens to survive or grow in foods. In an attempt to improve the microbiological safety of fish products, and in some instances to reduce spoilage while maintaining product quality, numerous non-thermal processing options are being investigated as alternative means of processing fish. These are reviewed below.

Ultraviolet light (UV)

UV has a wavelength of 200-400 nm and is lethal to bacteria and viruses within the 200-280 nm range, termed the UVC or germicidal range. The lethality of UV is predominantly due to the formation of thymine dimers which disrupt DNA structure and function in cells (USFDA 2001). UVC also produces ozone (O_3) which, as a free

radical species, oxidises microbial membranes and causes bacterial inactivation. UV has been used extensively on equipment and utensils in medical and food processing areas to reduce microbial contamination. Recently, UV irradiation has been used on meat and other food surfaces to directly reduce microbial loads. Studies relating to the use of UV on meat have indicated that this process does not change the colour or cause oxidative rancidity of the product (Stermer *et al* 1987; Wallner-Pendletone *et al* 1994). However, intense UV can cause the destruction of vitamins (particularly Vitamins B and C), oxidative deterioration of oils and fats leading to rancidity and, where fish oils are present, production of toxic by-products such as aldehydes. Operator hazards include eye complaints (e.g. conjunctivitis), erythema (i.e. reddening of the skin) and cancer, the risk of which should be minimised by wearing the appropriate protective apparel (e.g. glasses, gloves and long-sleeve clothing).

To achieve microbial inactivation, UV exposure of food must be $\geq$400 J/m^2 in all parts of the product. Other critical factors include transmissivity, the geometric configuration of the reactor, the power, wavelength and physical arrangement of the UV source, the product flow profile and radiation path (Stermer *et al* 1987; Wallner-Pendletone *et al* 1994). The use of a pulse power energisation technique (PPET), which applies UV light in pulses, greatly improves the peak power in the UV light source and, therefore, inactivates bacteria more rapidly than a continuous light source. As an example, PPET light sources operating at 1 pulse/second can reduce the cell population of *L. monocytogenes* by 10^6 CFU/unit in 512 μs (MacGregor *et al.* 1998).

Microorganisms can differ in their susceptibility to UV. For example, under the same set of conditions, *L. monocytogenes* showed the most resistance to UV, followed by *Staphylococcus aureus, Salmonella enteritidis, E. coli* and *Bacillus cereus*, in that order. Further experiments of UV-induced inactivation of *L.*

monocytogenes demonstrated that cells in a moist environment were killed more easily than those in a dry film or crust. In a practical sense, such results indicate that bacterial inactivation by UV will be more effective as an anti-microbial treatment of fish products when applied prior to their drying and smoking. While UV irradiation is yet to be used in the processing of fish, this method could be an option for in-line CSS prior to packaging. The installation of several UVC lamps above a belt before and/or after the slicing step might be appropriate and would be relatively inexpensive. Lamps could also be attached to the mobile slicing blade in the slicing machine as an alternative means of continuously sterilising the blades.

Irradiation

Ionising radiation within the context of the food industry is usually in the form of gamma rays, produced by radionuclides (e.g. cobalt, ^{60}Co or cesium ^{137}Cs), and newer technologies such as x-ray and e-beam irradiation. Gamma and x-ray irradiation can be used to treat thick foods as they penetrate several feet, whereas e-beam is effective only over several inches. Ionisation denatures cell membranes and enzymes required for survival and growth of microorganisms (Deeley 2002). The purpose of irradiation is not to sterilize the product, but to reduce the number of pathogens to a safer level. It can also be used to inactivate spoilage microorganisms and prolong shelf-life of fresh plant foods by decreasing the normal biological changes associated with growth and maturation processes. Within the three major micronutrients, lipids are those most affected by irradiation, resulting in oxidation. Proteins and carbohydrates are minimally affected. Vitamins are also minimally affected. Vacuum packaging helps to minimise these losses. Natural or microbial toxins in foods are unaffected by

irradiation and the use of high doses to combat high numbers of microorganisms is likely to reduce the organoleptic quality of the food.

Irradiation was introduced commercially in the 1960s and has since been approved for a number of foods in the US, as shown in Table 10. The International Atomic Energy Agency in conjunction with the World Health Organisation and Food and Agriculture Organisation convened a Joint Expert Committee on Food Irradiation that held a number of meetings from 1964 to 1980. The Joint Committee finally concluded that irradiation of any food commodity up to 10 KGy presents no toxicological or nutritional hazards in food. One study has shown that a dose of 2.5 to 3.7 KGy is required to eliminate five log units of *L. monocytogenes* from vacuum-packed RTE meats. Results vary with the product formulation. In a shelf-life study carried out by Kasimoğlu *et al* (2003), vacuum-packed sardines were irradiated at 1, 2 and 3 KGy doses. The shelf-life increased two- to threefold compared with the control samples. Lipid oxidation was observed on raw sardines, but not on cooked sardines.

Table 10 Foods approved for irradiation in the US (Source: CDC 2005)

Approval Year	Food	Dose (kGy)	Purpose
1963	Wheat flour	0.2-0.5	Control of mold
1964	White potatoes	0.05-0.15	Inhibit sprouting
1986	Pork	0.3-1.0	Kill *Trichina* parasites
1986	Fruit and vegetables	1.0	Insect control, increase shelf life
1986	Herbs and spices	30	Sterilisation
1990	Poultry	3	Bacterial pathogen reduction

1992	Poultry	1.5-3.0	Bacterial pathogen reduction
1997	Meat	4.5	Bacterial pathogen reduction
2000	Meat – unfrozen	4.5	Bacterial pathogen reduction
	Meat - frozen	7.0	

A combination of refrigeration temperatures and low irradiation doses, such as 1 to 2 KGy, could be used to treat VP-CSS to extend shelf-life and reduce the risk of illness from vegetative microbial pathogens. Unfortunately, installation costs are high with a commercial irradiation plant costing AU$2.5 million with an additional cost of AU$200K per year to run. Consumer acceptance is very important and traditionally this technology is not well accepted by consumers. In Australia, to date, sterilisation or pasteurisation by means of irradiation is limited to medical purposes (i.e. sterile dressings, needles, syringes and operating instruments), herbs, spices, herbal infusions, peanuts, cashew nuts, almonds and pistachio nuts. It is also used by The Australian Quarantine and Inspection Service to treat non-food "unknown weed seeds and plant parts" (Hahn 2002).

Pulsed Electric Fields

Pulsed electric field (PEF), also known as high electric field pulses, is an emerging technology that has been successfully applied in the preservation of fruit juices, purees, sauces, dairy and liquid poultry and egg products. The application of short pulses of very high electric fields (35-50 KV/cm) for microseconds is capable of inactivating pathogens, spoilage bacteria and some enzymes. In bacteria, PEF of sufficient intensity and duration causes irreversible damage to the membrane leading to "leakage" of important cellular components out of the cell and inactivation. Sub-

lethal injury to cells may occur if PEF treatment is not aggressive enough and it could allow bacteria to recover and grow.

Factors that affect PEF efficiency are, in decreasing order of importance, electric field strength, treatment time, number of pulses, pulse wave shape, processing temperature, type of microorganism, microbial growth phase, electrical conductivity of the food, ionic strength of the food, viscosity and pH of the food and presence of antimicrobials. Bacterial spores, Gram-positive cells and cells in stationary phase are more resistant to the effect of PEF than their vegetative, Gram-negative or logarithmic phase counterparts. Studies have shown that PEF efficiency can be improved by combining it with a elevated temperatures, for example in an experiment *L. monocytogenes* cells suspended in milk were treated with PEF at 25 and 50°C with the latter temperature yielding one log greater reduction. Currently this technology can only be applied to homogeneous (no air bubbles), low viscosity and high resistivity liquids making its use impossible to treat solid foods. Therefore, PEF is not a feasible method to inactivate pathogens, such a *L. monocytogenes,* and spoilage microorganisms in CSS.

Bacteriocins and Bacteriocin Producers

Bacteriocins are ribosomally-encoded antibiotic-like substances produced by specific strains of bacteria to inhibit the growth of other bacterial species. The main difference between traditional antibiotics and bacteriocins is that the latter have a limited bactericidal spectrum and are only toxic to bacteria closely related to the producing strain.

According to Klaenhammer (1993), 99% of all bacteria produce at least one bacteriocin. Gram-negative bacteria produce bacteriocins that are large and complex

proteins (29-90 kDa) which bind to specific receptors on the outer membrane of susceptible bacterial species. Gram-positive bacteria produce smaller bacteriocins (3-6 kDa) than the ones produced by Gram-negative bacteria and are classified as either lantibiotics (which are further divided into classes I, II or III) or non-lantibiotics. Lantibiotics are small peptides (<5 kDa) that contain the unusual amino acids lanthionine, α-methyllanthionine, dehydroalanine and dehydrobutyrine and kill susceptible microorganisms by depolarizing the cytoplasmic membrane, causing pore formation and "leakage" (type A) or by inhibiting the activity of cellular enzymes (type B). Most class I lantibiotics have a relatively broad inhibitory spectrum, i.e. they inhibit the growth of bacterial species that are closely related to the bacteriocin-producer, which in a natural environment will reduce competition, and may also inhibit other Gram-positive bacteria such as *L. monocytogenes, S. aureus, B. cereus and C. botulinum.* Because of their broad inhibitory spectrum, class I lantibiotics are of particular interest for their usefulness in food preservation.

The use of bacteriocins as antimicrobials in food systems is not new. Nisin, a Class I - type lantibiotic produced by the lactic acid bacteria (LAB) *Lactococcus lactis*, has been used as a food preservative (E234) since the 1950s and in 1988 FDA approved its use in pasteurized processed cheese spread. Up to now Nisin remains the most commercially important bacteriocin. It exhibits antimicrobial activity towards a wide range of Gram-positive bacteria and is particularly effective against spores (Delves-Broughton 2005).

Kim and Foegeding (1993) used Nisin in a spray form to evaluate its application on raw fillets of cod, herring and smoked mackerel inoculated with *Clostridium botulinum* type E spores. Their results showed a significant delay in toxin production at 10 and 26°C. Lactic acid bacteria, either by acid or by bacteriocin production, or

nutrient competition, may inhibit growth and toxin formation of *Clostridium botulinum*.

The growth of *L. monocytogenes* in VP-CSS treated with Nisin (400 and 1250 IU/g) was inhibited, but not prevented, when stored at 4°C for 21 days (Nilsson *et al* 1997; Szabo and Cahill 1999). Similar results were obtained when using the bacteriocin produced by *Pediococcus acidilatici* called pediocin (traded under the name ALTA^{TM} 2341) at 0.1 and 1%. The use of the bacteriocin producer *Carnobacterium divergens* V41 in CSS caused the level of *L. monocytogenes* to be unchanged from 50 CFU/g during four weeks of storage at 4 and 8°C (Brillet *et al* 2004).

Bacteriocins or bacteriocin producing bacteria, also known as protective cultures (PCs), could be added to VP-CSS to inhibit the growth of *L. monocytogenes* and other pathogens. Several technological and practical factors currently limit the use of PCs, including, but not limited to, effects of PCs on the sensory properties of the food in question, heat sensitivity of the PCs, inoculum size and lack of data on the interactions between PCs and the food poisoning or spoilage organisms (Rodgers *et al* 2002) or effects on consumers. The relatively recent discovery of substances, possibly proteins, produced by Antarctic Actinobacteria that inhibit the growth of *L. monocytogenes* and *S. aureus* (O'Brien *et al* 2004) may prove very useful in the application of bacteriocins in chill stored foods. Due to their adaptation to cold temperatures, it has been suggested that lantibiotics produced by Antarctic bacteria could be more active at low temperatures and will be advantageous when food products require storage below 4°C. Currently, none of the above mentioned bactericiocins, including Nisin, are approved to be used in seafood products in Australia and, as stated by Szabo and Cahill (1999), the regulatory approval for those

products could be a long and costly process, which should be considered by those interested in this technology.

High Pressure Processing

High pressure processing (HPP) consists of applying high pressures, for seconds or minutes at a time, varying from 100 to 1000 MPa (1MPa = 9.87 atm), to liquid or solid products packed in a flexible material. The item to be pressurised is submerged in a liquid, usually water, and the pressure increased to achieve the pre-set processing value. After a holding time, the vessel is depressurised back to the ambient pressure. Pressure generation is uniform and instantaneous throughout the chamber and products irrespective of size, shape or food composition. HPP appears to have little effect on the covalent bonds within the food, but ionic bonds can be disrupted resulting in unfolded proteins. HPP typically allows the retention of natural flavours, fragrances and nutrients such as vitamins. Other benefits of HPP include low energy consumption, minimum use of water, uniform action on the product and processing without chemical additives. Conversely, high implementation costs and relatively small batch sizes are the major negative factors for this technology.

Microbial cell membranes are the primary site of damage by HPP. Pressurised membranes show altered permeability leading to the loss of intracellular constituents and an inability to control the movement of substances into and out of the cell that results in inactivation. The conformation of proteins (including enzymes) may also be altered so that enzyme activity is enhanced, reduced or unaffected under pressure (Patterson 2004). Microorganisms show variable resistance to HPP, which is directly related to the amount of hydrostatic pressure applied. Stationary phase cells are more barotolerant than exponentially growing populations; spores are more tolerant than

Gram-positive cells, which are more tolerant than yeasts while Gram-negative bacteria show the least resistance to the lethal effect of HPP (Shigehisa *et al.* 1991).

Studies of *L. monocytogenes* inactivation by HPP have indicated that there is considerable variation between strains, with some capable of surviving and displaying a "tailing" behaviour after treatment with 800 MPa (Tay *et al* 2003).

Critical process factors for HPP are treatment pressure, time at pressure, the time to reach maximum pressure and to return to ambient pressure, treatment temperature, initial product temperature, distribution of vessel temperature at pressure, product pH, product composition, a_w of the product and packaging material integrity. Processing temperatures may range from <0 to >100°C. The temperature, pH and a_w profiles of HPP play an important role in inactivation of pathogens during that process.

Significance of HPP in seafood processing

The colour and texture of fish products are very important marketing factors and, after the product has been exposed to non-thermal treatment, they must remain as close as possible or be superior to their pre-processing state. Several studies have evaluated the use of HPP as a technology to inactivate pathogens, extend the shelf-life or produce seafood with a point of difference from that of competitors. Some products showed no textural change at all and in others large appearance and sensorial changes occur. HPP intensity and exposure time play a major role in these irreversible changes. In addition to the extrinsic factors described above there are also the intrinsic properties of the material subjected to HPP. Shucking oysters with 250-350 MPa for a maximum period of 3 minutes at ambient temperature is one of most successful commercial applications of HPP in North America (Cook, 2003). Those processing

conditions do not significantly change the raw material but are enough to inactivate the *Vibrio* spp, help with the meat detachment from the shell and also produce a "plumper" product with a better appearance.

Lakshmanan *et al* (2005) reported the effects of HPP (up to 300 MPa for up to 30 minutes) on cold smoked salmon colour, texture and changes in sensory attributes. Their results suggested that no more than 200 MPa for a period of 20 minutes can be applied without causing irreversible and unacceptable quality losses. The 200 MPa threshold has also been described by Fletcher *et al* (2005) as the point where unacceptable sensorial changes on raw Atlantic and king salmon fillets occur. Increased firmness, loss of translucency, increased drip loss and whitening are some of their major findings. Lakshmanan and Piggott (2003) also described unacceptable changes on pressure treated cold smoked salmon at 300 MPa. The cooked appearance and increased tissue firmness was attributed to the denaturation of myofibrilar and sarcoplasmic protein.

Conversely, thawed raw prawns treated with 400 MPa only showed slight but acceptable changes to their colour with no adverse textural modification noticed (Lopez-Caballero *et al* 2000). The authors of that study also proposed an increase of shelf-life of 14 days when 400 MPa was applied to vacuum packed samples.

Organic Acids and their Salts

A number of organic acids (and their salts) have been shown to have antimicrobial activity. Some, including lactates and diacetates, have been used extensively in the beef and poultry industries for several years and may prove useful within the seafood industry.

Sodium and potassium lactate at levels varying from 2 to 4% have a bacteriostatic effect on microbial populations (i.e. they increase the lag phase and reduce the rate of growth of populations) and this has been directly shown for a number of pathogenic bacteria including *L. monocytogenes, E. coli* O157:H7 and *Salmonella* spp. (Pelroy *et al* 1994; Yoon *et al* 2004). The effectiveness of these agents increases with increasing concentration and they are available commercially for application to foods in liquid form (60% w/w lactate) or powder (up to 96% w/w lactate). Liquid solutions are used more frequently because they can be mixed with brine and injected into the product. Both salts have been shown to decrease water activity sufficiently to slightly inhibit bacterial growth. Additionally, when in solution these salts dissociate, the ions react with free hydrogen ions in water and ultimately form lactic acid. As a weak acid, lactic acid has enhanced antimicrobial activity in that it can cross the bacterial membrane in its undissociated form and, thereby, reduce the pH of the cytoplasm. The cell will try to maintain its internal pH by neutralising or expelling protons but this slows the cell's growth (i.e. energy is diverted from growth-related functions).

Addition of 2% (w/w) sodium lactate solution (60% w/w) in comminuted, raw salmon showed that at 5°C, in combination with 3% water-phase salt (WPS, altered by the addition of NaCl) completely inhibited *L. monocytogenes* growth for up to 50 days. At 10°C the use of 3% sodium lactate, in combination with 3% WPS was required to achieve the same inhibition for up to 35 days (Pelroy *et al* 1994). Lactate is also capable of inhibiting non-proteolytic *C. botulinum* growth and, consequently, toxin production (Meng and Genigeorgis 1993). Two percent (2%) lactate in combination with 2% NaCl increased the lag phase of *C. botulinum* to 58 days when kept at less than 8°C.

A relatively new development in the application of organic salts in foods is the combination of sodium or potassium lactate with sodium diacetate. When mixed, these compounds exert a synergistic effect on each other and, thereby, improve the antimicrobial activity of the system.

Other salts that have been and continue to be investigated for their antimicrobial usefulness in fish products include sodium nitrite ($NaNO_2$) and sorbates. Pelroy *et al* (1994) has shown that $NaNO_2$ at 190-200 ppm inhibits the growth of *L. monocytogenes* more effectively in VP salmon compared to salmon packaged in oxygen-permeable film at 5 and 10°C. That level of sodium nitrite in combination with 5% WPS prevented the growth of *Listeria* for 34 days at 5°C. Nitrites are also efficient anti-botulinogenic compounds. However, the sensory influence of sodium nitrite in fish products awaits investigation, and there is also concern about the formation of nitrosamines which are potential carcinogens.

Neetoo *et al* (2008) have suggested the minimum inhibitory concentration (MIC) of sodium lactate, sodium diacetate, sodium benzoate and sodium sorbate to control *L. monocytogenes* growth on vacuum packed cold smoked salmon pate and fillets to be 4.6-5.6%, 0.11-0.22%, 0.25-0.5% and 0.38-0.75% respectively.

The use of organic acid salts in liquid or powder form to inhibit the growth of *L. monocytogenes* or other microorganisms of concern in fish products, including CSS, is worthy of investigation. The application of the antimicrobial agent could be incorporated into the curing step and, therefore, the exact method of application will depend on the curing procedure adopted (e.g. dry salting or brine injection). A problem anticipated in the application of a liquid form of an organic acid mixture is the uneven dispersion of the antimicrobial agent. This could be avoided by injecting the fillets with brine (and the antimicrobial solution) instead of dry salting the surface,

although the possibility of brine contamination and, thereby, the potential for inoculation of microorganisms inside the fish flesh should be considered. Thus, the brine solution to be injected should not be reused.

Modified Atmosphere Packaging

Modified atmosphere technology is the replacement of air (normally 21% oxygen, O_2; 79% nitrogen, N_2; and ~0.04% carbon dioxide, CO_2) with a specific mix of gases. The gaseous mixture inside the packaging changes, equilibrates and permeates within the product during storage but there can be no direct control or adjustment after the packaging step. Nitrogen is a filler gas to prevent pack collapse. The growth inhibition of aerobic microorganisms and reduction of oxidation are due to the absence of oxygen rather than the presence of nitrogen. For example, vacuum packaging also inhibits the growth of aerobic microorganisms and retards oxidation.

Carbon dioxide has bacteriostatic and fungistatic properties and is the most important gas used in MAP of fish (Sivertsvik *et al* 2002). The inhibitory effect of CO_2 in MAP is affected by composition, a_w and pH of the food, type of organism and concentration, volume of headspace and temperature (Daniels *et al* 1984). Generally, CO_2 has a greater effect on Gram-negative than on Gram-positive bacteria and is more inhibitory to the growth of microorganisms when its concentration is increased and as temperature or pH decrease. Other gases typically included in MAP are O_2 and N_2. The inclusion of O_2 maintains myoglobin in its oxygenated form, enhancing the typical red colour of fresh red meat (Brody 1989), including tuna, and N_2 is added as a replacement for O_2 to inhibit the growth of aerobic microorganisms and to retard the fat and/or oil oxidation that leads to rancidity (Farber 1990). Being an inert gas N_2 also helps to prevent pack collapse.

MAP technology has been studied extensively since the early 1930s, resulting in numerous research and review articles related to this topic (e.g. Sivertsvik *et al* 2002). Many researchers have concluded that MAP causes a "tremendous" increase in product shelf-life, whereas others report little or no shelf-life extension and this variability may be due to the different MAP compositions used or the food under investigation. In general, MAP extends the shelf-life of fresh seafood products by 30-60% (Sivertsvik *et al* 2002). In the context of fish products, Sivertsvik *et al* (2002) reported that MAP increases shelf-life compared to air packaging, but does not significantly improve the shelf-life in comparison to VP products. Relating specifically to the growth of *L. monocytogenes,* Nilsson *et al* (1997) showed that MAP of CSS with 70% CO_2 and 30% N_2 prevented the growth of *L. monocytogenes* for 8 days when stored at 5°C. Vacuum-packing of the same product resulted in a 5-log increase in the viability of *L. monocytogenes* during the same storage period, suggesting that MAP of fish is an effective means of reducing *L. monocytogenes* numbers.

As mentioned previously (*see* Section "*Microbial Spoilage*"), the atmosphere of storage of fish alters the SSO. The microorganisms able to grow in atmospheres imposed by MAP might further be inhibited by other processing options. For example, Emborg *et al* (2002) has demonstrated that on fresh MAP (60% CO_2 and 40% N_2) Atlantic salmon (*Salmo salar*) kept at 2°C *P. phosphoreum* dominated the spoilage biota and limited shelf-life to 14-21 days. Whereas if that product was frozen/thawed, *Carnobacterium pisicola* was the dominating biota and shelf-life was extended by 1-2 weeks. It was suggested that the freezing step eliminated *P. phosphoreum* and, therefore, the freezing of MAP fish products, such as raw salmon portions, might be of benefit to the extension of product shelf-life. The aim in

modifying the SSO by MAP of fish is to select for a species or a number of species that require more time to produce metabolites that lead to product rejection.

MAP can increase the shelf-life of fish products by 50-400% and reduces economic losses within the seafood industry due to spoiled products (Farber 1990). MAP also allows for longer distribution distances and a better quality product supplied to consumers. Negative aspects of MAP include the need for different formulations of gases for different products, strict temperature control, and the increased costs, compared to VP, in purchasing the gases and additional equipment required. Typically, the focus of MAP is to increase the shelf-life of the product by altering the SSO. There is some concern that the increase in shelf-life might lead to more food-borne illnesses caused by anaerobic or facultative anaerobic organisms. Of general concern is that some gas mixtures that extend the time to spoilage may not significantly suppress the growth and activity of pathogenic bacteria (Defernando *et al* 1995; Phillips 1996). In products with extended shelf lives stored at 4°C, *L. monocytogenes* or *C. botulinum* could grow and, thereby, cause illness in consumers. For example, *C. botulinum* toxin has been detected in MAP fish prior to products being considered spoiled (Post *et al* 1985; Garcia and Genigeorgis 1987; Taylor *et al* 1990). The packaging method best able to reduce non-proteolytic *C. botulinum* toxin production has been shown to be a mixture of equal parts of O_2 and CO_2. This "semi-aerobic" environment would impact the shelf-life of the product by allowing the growth of aerobic microorganisms and oil oxidation leading to rancidity, however, product safety must be of primary concern.

Raw Fish Treatments

A number of treatments to reduce spoilage and increase safety of fish products relate specifically to whole, raw, head-on, gilled and gutted (HOGG) fish or fillets of fish, rather than products with more processing options such as cold-smoked fish. For example, washing fish with a diluted chlorine solution can reduce the amount of bacteria on its surface. Chlorine at 10 ppm is considered safe by the USFDA. The use of a high pH treatment may also reduce the biota on fish. In a study by Gall (2005), food grade calcium hydroxide ($CaOH_2$) reduced *L. monocytogenes* numbers by ≥ 2 log units when HOGG salmon was left for 3 hours in limed water with a pH of 12.9. That process has been used commercially. Acidified sodium chlorite (ASC) is an antimicrobial agent approved by the USFDA for direct contact on seafood surfaces at concentrations of 40-50 ppm in water. Importantly, seafood to be eaten raw and treated with ASC must first be washed with potable water. ASC controls the growth of human pathogens but appears less effective on Gram-positive than Gram-negative bacteria.

Ozone, in its gaseous or liquid form, has been approved by the USFDA for direct contact on seafood, meat and poultry. Kim *et al* (2003) has described ozone as a strong microbiocidal agent against bacteria, fungi, parasites and viruses when these organisms are present in low ozone-demand media. Chlorine dioxide (ClO_2) is not specifically approved to be used on seafood, but is approved, in amounts equal or smaller than 3 ppm, as an antimicrobial agent in water to wash poultry, fruits and vegetables. It is more expensive than chlorine and requires an on-site generating system, but it is stable in a high organic environment. Steam surface pasteurization has also been suggested as a means of reducing *L. monocytogenes* numbers on fish.

Bremer and Osborne (1998) showed that that organism was reduced by four log units on king salmon when treated with steam for 8 seconds.

CONCLUSION

In summary, this chapter is intended to serve as background information and to 'set the scene' concerning the microbiological and technical issues, as well as applicability, of the non-thermal technologies discussed above to 'minimally processed' seafoods.

The following chapters consider the potential of non-thermal methods to extend the microbiological shelf-life and safety of lightly preserved seafoods.

REFERENCES

Anonymous. 1999. Haemolytic uraemic syndrome: a cluster of cases in early 1999. *New South Wales Public Health Bulletin* **10**:109-124.

Autio, T., Hielm, S., Miettinen, A., Sjöberg, M., Aarnisalo, K., Björkroth, J., Mattila-Sandholm, T. and Korkeala, H. 1999. Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout plant detected by pulsed-field gel electrophoresis typing. *Applied and Environmental Microbiology* **65**:150-155.

Barakat, R.K. and Harris, L.J. 1999. Growth of *Listeria monocytogenes* and *Yersinia enterolitica* on cooked modified-atmosphere-packaged poultry in the presence and absence of a naturally occurring microbiota. *Applied and Environmental Microbiology* **65**:342-345.

Basby, M., Jeppesen, V.F. and Huss, H.H. 1998. Characterization of the microflora of lightly salter lumpfish (*Cyclopterus* lumpus) roe stored at 5°C. *Journal of Aquatic Food Product Technology* **7**:35-51.

Bremer, P. and Osborne, C. 1998. Reducing total aerobic counts and *Listeria monocytogenes* on the surface of king salmon (*Oncorhynchus tshawytscha*). *Journal of Food Protection* **61**:849-854.

Brillet, A., Pilet, M.-F., Prevost, H., Bouttefroy, A. and Leroi, F. 2004. Biodiversity of *Listeria monocytogenes* sensitivity to bacteriocin-producing *Carnobacterium* starins and application in sterile cold smoked salmon. *Journal of Applied Microbiology* **97**:1029-1037.

Brody, A.L. 1989. Modified atmosphere/vacuum packaging of meat, pp 1-37. *In* Brody, A.L. (ed). Controlled/modified atmosphere/vacuum packaging of foods. Food and Nutrition Press, Trumbull.

Cahill, M.M. 1990. Bacterial flora of fishes: a review. *Microbial Ecology* **19**:21-41.

CDC (Center for Disease Control). 2005. Food irradiation (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/foodirradiation.htm - accessed February 2006).

Christian, J.H.B. 1971. The ecology of *Clostridium botulinum* type E in the Australian environment. Technical reports Series No. 125. IAEA research contracts. Eleventh Annual Report. Vienna: International Atomic Energy Agency 96-97.

Cook, D.W. 2003. Sensitivity of *Vibrio* species in phosphate-buffered saline and in oysters to high-pressure processing. *Journal of food protection* **66**:2276-2282.

Cook, D.W., O'Leary, P., Hunsucker, J.C., Sloan, E.M., Bowers, J.C., Blodgett, R.J. and DePaola, A. 2002. *Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S. retail shell oysters: a national survey from June 1998 to July 1999. *Journal of Food Protection* **65**:79-87.

Dalgaard, P. 2000. Freshness, quality and safety in seafoods (seafood.ucdavis.edu/pubs/qualitysafety.doc – accessed February 2006). University of California.

Dalgaard, P. 2002. Modelling and prediction of the shelf-life of seafood, pp 191-219. *In* Safety and quality issues in fish processing. Bremner, H.A. (ed), Woodhead Publishing, Cambridge.

Dalgaard, P. 2006. Microbiology of marine muscle foods, pp53-1-53-20. *In* Handbook of food science, technology and engineering. Hui, Y.H. (ed), CRC Press, Boca Raton.

Dalgaard, P., Gram, L. and Huss, H.H. 1993. Spoilage and shelf life of cod fillets packed in vacuum or modified atmosphere. *International Journal of Food Microbiology* **19**:283-294.

Dalgaard, P., Mejlholm, O., Christiansen, T.J. and Huss, H.H. 1997. Importance of *Photobacterium phosphoreum* in relation to spoilage of modified atmosphere-packed fish products. *Letters in Applied Microbiology* **24**:373-378.

D'Aoust, J.-Y. 2000. *Salmonella*, pp 1233-1299. *In* Lund, B.M., Baird-Parker, T.C., Gould, G.W. (ed). The microbiological safety and quality of food, Aspen Publishers, Gaithersburg.

Daniels, J., Krishnamurthi, R. and Rizvi, S. 1984. A review of effects of carbon dioxide on microbial growth and food quality. *Journal of Food Protection* **48**:532:537.

Defernando, G., Nychas, G., Peck, M. and Ordonez, J. 1995. Growth survival of psychrotrophic pathogens on meat packaged under modified atmosphere. *International Journal of Food Microbiology* **28**:221-231.

Deeley, C. 2002. Food irradiation: setting new standards or a slippery slope? *Food Science and Technology* **16**:52-55.

Delves-Broughton, J. 2005. Nisin as a food preservative. *Food Australia* **57**:525-527.

Dillon, R., Patel, T. and Ratman, S. 1994. Occurrence of *Listeria* in hot and smoked salmon seafood products. *International Journal of Food Microbiology* **22**:73-77.

Dodds, K.L. 1993. *Clostridium botulinum* in the environment, pp 21-52. *In Clostridium botulinum*. Hauschild, H.W. and Dodds, K.L. (ed.), Marcel Dekker, New York.

Dufresne, I., Smith, J.P., Jiun, N.L. and Tarte, I. 2000. Effect of headspace oxygen and film of different oxygen transmission rate on toxin production by *Clostridium botulinum* type E in rainbow trout stored under modified atmospheres. *Journal of Food Safety* **20**:157-175.

Eklund, M.W., Poysky, F.T., Paranjpye, R.N., Lashbrook, L.C., Peterson, M.E. and Pelroy, G.A. 1995. Incidence and sources of *Listeria monocytogenes* in cold smoked salmon fishery products and processing plants. *Journal of Food Protection* **58**:502-508.

Emborg, J., Laursen, B., Rathjen, T. and Dalgaard, P. 2002. Microbial spoilage and formation of biogenic amines in fresh and thawed modified atmosphere-packed salmon (*Salmo salar*) at 2°C. *Journal of Applied Microbiology* **92**:790-799.

Emborg J., Laursen, B. and Dalgaard, P. 2005. Significant histamine formation in tuna (*Thunnus albacares*) at 2 °C - effect of vacuum- and modified atmosphere-packaging on psychrotolerant bacteria. *International Journal of Food Microbiology* **101**:263-279.

European Parliament and Council. 1995. Directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners. Official Journal of the European Communities **38**:24.

Faith, N.G., Yousef, A.E. and Luchansky, J.B. 1992. Inhibition of *Listeria monocytogenes* by liquid smoke and isougenol, a phenolic component found in smoke. *Journal of Food Safety* **12**:303-314.

Farber, J.M. 1990. Microbiological aspects of modified atmosphere technology – a review. *Journal of Food Protection* **54**:58-70.

Farber, J.M. 1991. *Listeria monocytogenes* in fish products. *Journal of Food Protection* **54**:922-924.

FDA/ORA (US Food and Drug Administration/US Office of Regulatory Affairs). 1996. Tuna, Sable, Salmon, Shad – smoked cured, adulteration involving food additives, sodium nitrite. Compliance Policy Guides Manual Sec. 540.500.

Fletcher, G., Tume, R.K., Alvin, L. and Ng, S.Y. 2005. The effect of high pressure processing on fresh Atlantic and king salmon fillets. Second Innovative Foods Centre Conference and the IFT / EFFoST. Non-Thermal Food Processing Technologies Workshop, Food Science Australia, Sydney, *14 - 17 September*

FSANZ (Food Standards Australia and New Zealand). 2006. Australia New Zealand Food Standards Code. Anstat, Canberra.

Gall, K. 2005. Seafood industry collaboration advances *Listeria* control in ready-to-eat seafood plants. *Food Safety Magazine* August/September. http://www.foodsafetymagazine.com/article.asp?id=303&sub=sub1

Garcia, G. and Genigeorgis, C. 1987. Quantitative evaluation of *Clostridium botulinum* nonproteolytic type B, type E, and type F growth risk in fresh salmon tissue homogenates stored under modified atmospheres. *Journal of Food Protection* **50**:390.

Garland, C.D. 1995. Microbiological quality of aquaculture products with special reference to *Listeria monocytogenes* in Atlantic salmon. *Food Australia* **47**:559-563.

Gennari, M. and Tomaselli, S. 1988. Changes in aerobic microflora of skin and gills of Mediterranean sardines (*Sardinus pilchardus*) during storage in ice. *International Journal of Food Microbiology* **6**:341-347.

Gennari, M., Tomaselli, S. and Cotrona, V. 1999. The microflora of fresh and spoiled sardines (*Sardina pilchardus*) caught in Adriatic (Mediterranean) Sea and stored in ice. *Food Microbiology* **16**:15-28.

Gram, L. 1991. Inhibtion of mesophilic spoilage by *Aeromonas* spp. on fish by salt, potassium sorbate, liquid smoke, and chilling. *Journal of Food Protection* **54**:436-442.

Gram, L. 2001. Potential hazards in cold-smoked fish: *Clostridium botulinum* type E. *Journal of Food Science* **66**:1082-S-1087.

Gram, L. 2006. Microbial food spoilage, pp 51-1 to 51-16. *In* Handbook of food science, technology and engineering. Hui, Y.H. (ed), CRC Press, Boca Raton.

Gram, L. and Huss, H.H. 2000. Fresh and processed fish and shellfish, pp 472-506. *In* Lund, B.M., Baird-Parker, T.C., Gould, G.W. (ed.), The microbiological safety and quality of food. Aspen Publishers, Gaithersburg.

Guldager, H.S., Bøknæs, N., Østerberg, C., Nielsen, J. and Dalgaard, P. 1998. Thawed cod fillets spoil less rapidly than unfrozen fillets when stored under modified atmosphere at 2°C. Journal of Food Protection **61**:1129-1136.

Hahn, S. 2002. The use of food irradiation in Australia. Proceedings of the 2nd New Zealand HACCP Conference, Christchurch, New Zealand.

Hauschild, A.H.W. 1993. Epidemiology of human botulism. Pp. 69-104. *In* *Clostridium botulinum*: ecology and control in foods. A.W. Hauschild and K.L. Dodds (ed). Marcel Dekker, New York.

Hilderbrand, K.S. 1991. Quick determination of water phase salt content of cold smoked fish. Oregon State University, Extension Sea Grant, Special Report 883, August.

Hudson, J.A., Mott, S.J., Delacy, K.M. and Edridge, A.L. 1992. Incidence and coincidence of *Listeria* spp., motile aeromonads and *Yersinia enterolitica* on ready-to-eat fleshfoods. *International Journal of Food Microbiology* **16**:99-108.

Huss, H.H. 1981. *Clostridium botulinum* type E and botulism. D.Sc Thesis. Technical University of Denmark, Denmark.

Huss, H.H. 1993. Assurance of seafood quality. FAO Fisheries Technical Paper **334**:1-164 (web access: http://www.fao.org/DOCREP/003/T1768E/T1768E07.htm#ch6).

Huss, H.H. 1995. Quality and quality changes in fresh fish. FAO Fisheries Technical Paper **348**:1-195.

Huss, H.H., Ababouch, L. and Gram, L. 2003. Assessment and management of seafood safety and quality. FAO Fisheries Technical Paper **444**:1-230.

ICMSF (International Commission on Microbiological Specifications for Foods). 1986. *Microorganisms in Foods 2, Sampling For Microbiological Analysis: Principles and Specific Applications*, 2nd ed. University of Toronto Press, Buffalo, NY.

Jay, J.M. 1996. Microorganisms in fresh ground meats: relative safety of products with low versus high numbers. *Meat Science* **43**:S59-S66.

Jay, J.M. 1997. Do background microorganisms play a role in the safety of fresh foods? *Trends in Food Science and Technology* **8**:421-424.

Jørgensen, L.V. and Huss, H.H. 1998. Prevalence and growth of *Listeria monocytogenes* in naturally contaminated seafood. *International Journal of Food Microbiology* **42**:127-131.

Jørgensen, L.V., Dalgaard, P. and Huss, H.H. 2000. Multiple quality index for cold-smoked salmon (*Salmo salar*) developed by multivariate regression of biogenic amines and pH. *Journal of Agricultural and Food Chemistry* **48**:2448-2453.

Kanuganti, S.R., Wesley, I.V., Reddy, P.G., Mckean, J. and Hurd, H.S. 2002. Detection of *Listeria monocytogenes* in pigs and pork. *Journal of Food Protection* **65**:1470-1474.

Kasimoğlu, A., Denli, E. and İç, E. 2003. The extension of the shelf-life of sardine which were packaged in a vacuum and stored under refrigeration. *International Journal of Food Science and Technology* **38**:529-535.

Kaysner, C.A. 2000. *Vibrio* species, pp 1336-1362. *In* The microbiological safety and quality of fodd. Lund, B.W., Baird-Parker, T.C. and Gould, G.W. (ed.), Aspen Publishers, Gaithersburg.

Kim, J. and Foegeding, P.M. 1993. Principles of control, pp121-176. *In* Hauschild, A.H.W., Dodds, K.L (ed), *Clostridium botulinum*: ecology and control in foods, Marcel Dekker, New York.

Kim, J., Yousef, A. and Khader, M. 2003. Ozone and its current and future application in the food industry. *Advances in Food Nutrition Research* **45**:167-218.

Klaenhammer, T.R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. *Federation of European Microbiological Societies Microbiology Reviews* **12**:39-85.

Kraa, E. 1995. Surveillance and epidemiology of foodborne illness in NSW, Australia. *Food Australia*. **47**:418-423.

Lakshmanan, R.and Piggott, J.R. 2003. High-pressure processing of cold-smoked salmon. First Joint Trans-Atlantic Fisheries Technology Conference. 33rd WEFTA and 48th AFTC meetings, 11-14 June, Reykjavik, Iceland

Lakshmanan, R., Miskin, D. and Piggott, J.R. 2005. Quality of vacuum packed cold-smoked salmon during refrigerated storage as affected by high-pressure processing. *J. Sci. Food Agric.*, **85**:655-661

Larsen, E., Hyldig, G., Dalgaard, P., Bech, A.C. and Østerberg, C. 2003. Consumers and experts responses to fresh cold fillets, pp 345-360. *In* Luten, J.B., Oehlenschlager, J., Ólafsdóttir, G. (ed.), Quality of fish from catch to consumer. Wageningen Academic Publishers, Wageningen.

Lehane, L. and Olley, J. 2000. Histamine fish poisoning revisited. *International Journal of Food Microbiology* **58**:1-37.

Leroi, F., Joffraudd, J.J., Chevallier, F. and Cardinal, M. 1998. Study of the microbial ecology of cold smoked salmon during storage at 8°C. *International Journal of Food Microbiology* **39**:111-121.

Leroi, F., Joffraud, J.J. and Chevallier, F. 2000. Effect of salt and smoke on the microbiological quality of cold smoked salmon during storage at 5°C as estimated by the factorial design method. *Journal of Food Protection* **63**:502-508.

Leroi, F. and Joffraud, J.J. 2000. Salt and smoke simultaneously affect chemical and sensory quality of cold smoked salmon during 5°C storage. *Journal of Food Protection* **63**:1222-1227.

Lopez-Caballero, M.E., Perez-Mateos, M., Borderias, J.A. and Montero, P. 2000. Extension of the shelf life of prawns (*Penaeus japonicus*) by vacuum packaging and high-pressure treatment. *Journal of Food Protection*, **63**:1381-1388

Lorca, T.A., Pierson, M.D., Flick, G.J. and Hackney, C.R. 2001. Levels of *Vibrio vulnificus* and organoleptic quality of raw shellstock oysters (*Crassostrea virginica*) maintained at different storage temperatures. *Journal of food protection* **64**:1716-1721.

Lund, B.M. and Peck, M.W. 2000. *Clostridium botulinum*, pp 1057-1109. *In* The microbiological safety and quality of food. Lund, B.M., Baird-Parker, T.C. and Gould, G. (ed.), Aspen Publishers, Gaithersburg.

McLauchlin, J. 1997. The pathogenicity of *Listeria monocytogenes*: a public health perspective. *Reviews in Medical Microbiology* **8**:1-14.

MacGregor, S.J, Rowan, N.J., McIlvaney, L., Anderson, J.G. and Farish, O. 1998. Light inactivation of food-related pathogenic bacteria using power source. *Letters in Applied Microbiology* **27**:67-70.

McMeekin, T.A., Olley, J., Ratkowsky, D.A. and Ross, T. (1993). *Predictive Microbiology:Theory and Application.* Research Studies Press, Taunton, UK.

Mejlholm, O. and Dalgaard, P. 2002. Antimicrobial affect of essential oils on the seafood spoilage microorganism *Photobacterium phosphoreum* in liquid media and fish products. *Letters in Applied Microbiology* **34**:27-34.

Mejlholm, O., and Dalgaard, P. (2007). Modeling and predicting the growth boundary of *Listeria monocytogenes* in lightly preserved seafood. *Journal of Food Protection,* 70: 70-84

Meng, J. and Genigeorgis, C.A. 1993. Modeling lag phase of non-proteolytic *Clostridium botulinum* toxigenesis in cooked turkey and chicken breasts as affected by temperature, sodium lactate, sodium chloride and spore inoculum. *International Journal of Food Microbiology* **19**:109-122.

Messina, M.C., Ahmad, H.A., Marcello, J.A., Gerba, C.P. and Paquette, M.W. 1988. The effect of liquid smoke on *Listeria monocytogenes. Journal of Food Protection* **51**:629-631.

Neetoo, H., Ye, M. and Chen, H.Q. 2008. Potential antimicrobials to control *Listeria monocytogenes* in vacuum-packaged cold-smoked salmon fillets and pate. *International Journal of Food Microbiology* **123**:220-227.

Nickelson, R. and Finne, G. 1992. Fish, crustaceans, and precooked seafoods, pp 875-895. *In* Compendium of Methods for the Microbiological Examination of Foods. Vanderzant, C. and Splittstoesser, C.F. (ed), American Public Health Association, Washington, DC.

Niedziela, J.C., Macrae, M., Ogden, I.D. and Nesvadba, P. 1998. Control of *Listeria monocytogenes* in salmon: antimicrobial effect of salting, smoking and specific smoke compounds. *Lebensmittel Wissenschaft und Technologie* **31**:155-161.

Nilsson, L., Huss, H.H. and Gram, L. 1997. Inhibition of *Listeria monocytogenes* on cold-smoked salmon by nisin and carbon dioxide atmosphere. *International Journal of Microbiology* **38**:217-27.

O'Brien, A., Sharp, R., Russell, N.J. and Roller, S. 2004. Antarctic bacteria inhibit growth of food-borne microorganisms at low temperatures. *FEMS Microbiology Ecology* **48**:157-167.

Paarup, T., Sanchez, J.A., Moral, A., Christensen, H., Bisgaard, M. and Gram, L. 2002. Sensory, chemical and bacteriological changes during storage of iced squid (*Todaropsis eblanae*). *Journal of Applied Microbiology* **92**:941-950.

Patterson, M. 2004. Under pressure: a novel technology to kill micro-organisms in foods. *Culture magazine* **24**:2.

Pelroy, G., Peterson, M., Paranjpye, R., Almond, J. and Eklund, M. 1994. Inhibition of *Listeria monocytogenes* in cold-process (smoked) salmon by sodium nitrite and packaging material. *Journal of Food Protection* **57**:114-119.

Phillips, C. 1996. Modified atmosphere packaging and its effects on the microbiological quality and safety of produce. *International Journal of Food Science and Technology* **31**:463-479.

Post, L.S., Amoroso, T.L. and Solberg, M. 1985. Inhibition of *Clostridium botulinum* type E in model acidified food systems. *Journal of Food Science* **50**:966-968.

Price, R.J. and Tom, P.D. 2003. Compendium of fish and fishery product processes, hazards and controls,

(http://seafood.ucdavis.edu/HACCP/compendium/compend.htm). Food Science and Technology, University of California (Davis).

Rodgers, S., Kailasapathy, K., Cox, J. and Peiris, P. 2002. Bacteriocin production by protective cultures. *Food Science Technology* **2**:59-68.

Rørvik, L.M, Caugant, D.A. and Yndestad, M. 1995. Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. *International Journal of Food Microbiology* **25**:19-27.

Rørvik, L.M., Yndestad, M. And Skjerve, E. 1991. Growth of *Listeria monocytogenes* in vacuum packed smoked salmon during storage at 4°C. *International Journal of Food Microbiology* **14**:111-118.

Ross, T. and Sanderson, K. 2000. A risk assessment of selected seafoods in NSW. Report prepared for SafeFood NSW. University of Tasmania, Hobart.

Shigehisa, T., Ohmori, T., Saito, A., Taji, S. and Hayashi, R. 1991. Effects of high hydrostatic pressure on characteristics of pork slurries and inactivation of microorganisms associated with meat and meat products. *Int. J. Microbiol.* **12**:207-216.

Singh, T.K. and Cadwallader, K.R. 2003. The shelf life of foods: an overview. *Freshness and shelf life of foods ACS symposium series* **836**:2-21.

Sivertsvik, M., Jeksrud, W. and Rosnes, J.T. 2002. A review of modified atmosphere packaging of fish and fishery products – significance of microbial growth, activities and safety. *International Journal of Food Science and Technology* **37**:107-127.

Stermer, R.A, Lasater-Smith, M. and Brasington, C.F. 1987. Ultraviolet radiation – an effective bactericide for frsh meat. *Journal of Food Protection.* **50**:108-111.

Szabo, E.A. and Cahill, M.E. 1999. Nisin and ALTA^{TM} 2341 inhibit the growth of *Listeria monocytogenes* on smoked salmon packaged under vacuum or 100% CO_2. *Letters in Applied Microbiology* **28**:373-377.

Suňen, E. 1998. Minimum inhibitory concentration of wood smoke extracts against spoilage and pathogenic micro-organisms associated with foods. *Letters in Applied Microbiology* **27**:45-48.

Tay, A., Shellhammer T.H., Yousef A.E. and Chism, G.W. 2003. Pressure death and tailing behavior of *Listeria monocytogenes* strains having different barotolerances. *Journal of Food Protection* **66**:2057-2061.

Taylor, L.Y., Cann, D.D. and Welch, B.J. 1990. Antibotulinal properties of nisin in fresh fish packaged in an atmosphere of carbon dioxide. *Journal of Food Protection* **53**:953-957.

Thurette, J., Membre, J.M., Ching, L.H., Tailliez, R. and Catteau, M. 1998. Behavior of *Listeria* spp. in smoked fish products affected by liquid smoke, NaCl concentration, and temperature. *Journal of Food Protection* **61**:1475-1479.

Truesltrup Hansen, L. 1995. Quality of chilled vacuum packed cold smoked salmon. PhD thesis. Royal Veterinary and Agricultural University, Frederiksberg, Denmark.

Tryfinopoulou, P., Tsakalidou, E. and Nychas, G-J.E. 2002. Characterization of *Pseudomonas* spp. associated with spoilage of gilt-head sea bream stored under various conditions. *Applied and Environmental Microbiology* **68**:65-72.

USFDA (United States Food and Drug Administration). 2001. Processing parameters needed to control pathogens in cold smoked fish

(http://www.cfsan.fda.gov/~comm/ift2cont.html - accessed February 2006). Center for Food Safety and Applied Nutrition.

Valenti, M., Daubert, S. Bucellati, L. and Cantoni, C. 1991. *Listeria* spp. in salmons. *Industrie Alimentari* **30**:741-743.

Wallner-Pendleton, E.A, Summer, S.S., Froning, G.W. and Stetson, L.E. 1994. The use of ultraviolet radiation to reduce *Salmonella* and psychrotrophic bacterial contamination on poultry carcasses. *Poultry Science* **73**:1327-1333.

Yoon K.S., Burnette C.N., Abou-Zeid K.A. and Whiting R.C. 2004. Control of growth and survival of *Listeria monocytogenes* on smoked salmon by combined potassium lactate and sodium diacetate and freezing stress during refrigeration and frozen storage. *Journal of Food Protection* **67**:2465-2471.

Chapter 2: Comparative assessment of three commercial antimicrobial compounds used to inhibit *Listeria monocytogenes* growth on cold smoked salmon and their influence on the sensorial properties and shelf-life of the final product

INTRODUCTION

Chilled vacuum packed cold smoked salmon

Chilled vacuum packed cold smoked salmon (CVPCSS) is a recent product that originated in France during the 1920s. World consumption was estimated to be around 70000 metric tones with an aggregated value of US$ 950 million annually (Jørgensen *et al.* 2000).

CVPCSS is a ready-to-eat product and as such does not require any further heat treatment prior to consumption. By definition, cold-smoked salmon has not been exposed to any intense (i.e. bactericidal) heat (>30°C) during processing making, it a high risk product for the YOPIs (young, old, pregnant and immuno-compromised individuals) because of the potential presence and growth of the pathogen *Listeria monocytogenes (see* next Section) on the product (FAO/WHO, 2004).

Characteristics of cold smoked salmon include 3-8% water phase salt (WPS) corresponding to water activities of 0.950-0.983, pH of 5.9-6.3 and 6.7-11 g/l water phase lactate (Dalgaard and Jørgensen 1998; *see also* Table 2, Chapter 1) with shelf-life ranging from 2 to 12 weeks when stored at 5°C, depending on the manufacturer and processing parameters. Leroi *et al* (2000) and Leroi and Joffraud (2000) have shown that the concentration of smoke components and salt are very important factors that influence microflora development and shelf-life of cold smoked salmon.

When vacuum packed and kept below 10°C spoilage is primarily due to microbial activity. Lactic acid bacteria (LAB) are the main spoilage bacteria but often *Photobacterium phosphoreum* and/or Enterobacteriaceae are also found.

Total count at the sensory rejection point is around 10^7-10^8 cfu/g and microflora isolated at this point appears to differ considerably from one study to another. Different processing parameters and microbiological methods are possibly the major sources of variation (Truelstrup Hansen *et al* 1998). The off-flavours and odours developed in chilled vacuum packed cold smoked salmon have been described as sour, acid, pungent and occasionally faecal (Truesltrup Hansen 1995; Leroi *et al* 1998).

Listeria monocytogenes

As noted in Chapter 1, *L. monocytogenes* is the only human pathogenic strain of the *Listeria* genus. Although, several studies have suggested that 10% of the healthy individuals may carry this pathogen in their intestinal tract without being affected by it. It acts as an opportunistic pathogen for both humans and animals causing abortions, neonatal sepsis, severe infections such as septicaemia and meningoencephalitis in susceptible hosts (YOPIs). In these groups the mortality from listeriosis can be as high as 30% (McLauchlin 1997). In the US alone it is estimated to cause approximately 2500 cases of human illness and 500 deaths annually (Kanuganti *et al* 2002).

Its growth limiting conditions are very wide. It can grow with or without oxygen, at min. a_W of 0.92, pH of 4.4 – 9.4, temperature of -0.4 - 45°C and maximum 14% NaCl. As a consequence it is capable of growing in many perishable products with extended shelf-life (Barakat and Harris 1999; Rørvik *et al* 1991) and presents a serious risk

especially in those products that do not require bactericidal heat treatment by the consumer prior to consumption (e.g., chilled vacuum packed cold smoked salmon).

The incidence of *L. monocytogenes* in VPCSS and cooked fish products has been reported to range from 4.3 to 36% but a comprehensive risk assessment (USDA/FDA 2001) estimated that an average of 15% of all smoked fish is contaminated with this bacterium. Farber (1991) and Hudson *et al* (1992) reported a contamination rate of between 20 and 80% of *L. monocytogenes* in cold smoked salmon whereas Valenti *et al* (1991) and Dillon *et al* (1994) could not find any *L. monocytogenes* in the few investigations they performed. Jørgensen and Huss (1998) found that 34% of freshly produced cold smoked salmon manufactured by Danish processors were contaminated with *L. Monocytogenes.* This percentage increased to 43% after the product was kept at 5°C for 20-50 days. Tables 1, 2 and 3 show the prevalence of *Listeria spp* and/or *L. monocytogenes* in the environment, fresh, and cold smoked fish respectively, confirming their presence on these raw and finished products and indicating that a possible risk to consumers may exist.

Rørvik *et al* (1995), Eklund *et al* (1995) and Jørgensen and Huss (1998) suggested that the natural level of *L. monocytogenes* in freshly produced cold smoked salmon is, in most cases, less than 10 cfu/g and more rarely between 10 and 100 cfu/g. Jørgensen and Huss (1998) also estimated that contamination levels of less then 10, 100 and 1000 cfu/g were found respectively in 28, 5 and 1% of the freshly processed cold smoked salmon samples.

Table 1 Prevalence of *Listeria* spp. in freshwater, seawater and sediments (*after* USDA/FDA, 2001)

Sampling location	**No. of Samples**	**% positive for**	
		***Listeria* spp.**	***L. monocytogenes***
Freshwater			
river, domestic animals (USA)	37	81	62
not specified (UK)	7	100	nd
river, populated (UK)	36	nd	47
canals, lakes (Holland)	180	nd	37
ground water (Switzerland)	12	0	0
spring water (Switzerland)	12	0	0
Seawater			
coastal area (USA)	3	33	33
shellfish growing area (USA)	70	3	nd
not specified (Holland)	43	0	0
used for salmon transport (Norway)	21	52	14
around salmon farm (Norway)	8	0	0
Sediments			
freshwater (USA)	46	30	17
freshwater (USA)	15	20	0

nd = not determined

Ben Embarek (1994) and Eklund *et al* (1995) have suggested that *L.monocytogenes* may be present in raw salmon in low numbers because the seawater from estuarine environments and costal areas where they grow are constantly exposed to potential

contamination with this bacterium. Their observations are reinforced by the data summarized in Table 1, above.

Table 2 Prevalence of *Listeria* spp. and *Listeria monocytogenes* in live or newly slaughtered fish (*after* USDA/FDA, 2001)

Sampling location	**No. of samples**	**% positive for**	
		***Listeria* spp.**	***L. monocytogenes***
Freshwater			
skin of live trout (Switzerland)	45	33	11
channel catfish (USA)	4	100	nd
slaughtered trout (Switzerland)	27	22	15
Seawater			
salmon, at harvest (Norway)	10	0	0
salmon, at processing plant (Norway)	18	0	0
salmon (Faroe islands)	18	nd	1
frozen salmon (received at plant) (USA)	65	nd	34
salmon (USA, Chile, Norway, Canada, Scotland)	32	nd	10

nd = not determined

Table 3 Prevalence of *Listeria* spp. *and L. monocytogenes* in cold-smoked fish (*after* USDA/FDA, 2001)

Sampling location	**No. of samples**	**% positive for**	
		***Listeria* spp.**	***L. monocytogenes***
Salmon			
Norway	33	nd	9
Norway	40	80	33
Norway	65	11	11
Italy	63	0-100	0-29
Italy	165	nd	19
Switzerland	100	nd	24
Switzerland	64	nd	6
New Zealand	12	nd	75
UK	22	14	nd
USA	61	nd	79
Iceland	13	23	0
Canada, USA, Chile, Scotland, Norway	32	nd	31
Japan	76	30	16
Denmark	188	nd	34
Trout			
Switzerland	49	4	2

nd = not determined

From molecular typing studies, Rørvik *et al* (1995) and Autio *et al* (1999) considered that raw salmon and trout are not important sources of *Listeria monocytogenes* contamination in cold smoked fish because the type of the *L. monocytogenes* in the raw material differs from the type in the cold smoked salmon. This suggests that the indigenous *L. monocytogenes* in the raw salmon will be eliminated and then replaced, during production via cross-contamination, by other *L. monocytogenes* types. The recontamination is likely to come from niches of *L. monocytogenes* (ie, injectors, biofilms in tables, belts, slicers, cutting boards, etc...) that survive the sanitation step.

Cold Smoke Processing

Cold smoke processing is characterized by a combination of salting/curing prior to the smoking (although some producers also add sucrose to their wet/dry salting methods). The use of nitrite salts for cold smoked salmon processing is allowed in US (220 ppm $NaNO_2$ maximum) but not in Europe (European Parliament and Council 1995; FDA/ORA 1996).

Smoking time, temperature and relative humidity can vary from manufacturer to manufacturer and may range from 8–48 h, 15-30°C and 50-85% respectively (Truelstrup Hansen 1995; Rørå *et al.* 1998; Jørgensen *et al* 2000). These mild temperatures are not sufficient to denature the fish protein nor to kill or reduce significantly the number of spoilage microorganisms. This is very important as they will compete for nutrients against pathogens, ultimately limiting their growth (e.g., *L. monocytogenes* and *Clostridium botulinum*), toxin production (e.g., *C. botulinum*) and germination (spores of *C. botulinum*) (Price and Tom 2003).

The addition of sodium chloride (NaCl, table salt) to the salmon lowers the water activity (a_w) in the salmon flesh reducing its microbial activity and enhancing

product safety. The water phase-salt (WPS) is the determining factor for water activity of sliced cold smoked salmon (Huss *et al* 1995; Jørgensen *et al* 2000) and can be calculated by the following equation (Hilderbrand 1991):

$$WPS = (\% \text{ of NaCl} \times 100) / (\% \text{ of NaCl} + \% \text{ of } H_2O)$$

or

$$WPS = (NaCl\ (g) \times 100\%) / (NaCl\ (g) + H_2O\ (g)),$$

where (g) = weight in grams of salt or water per 100g of product.

The above calculations require the knowledge of percentage or mass of both salt and moisture in the product. Alternatively, Jørgensen *et al* (2000) have estimated the relationship between WPS and a_w for cold smoked salmon to be described by the following equation:

$$a_w = 1 - (0.005 \times WPS) - (1.3 \times 10^{-4} \times WPS^2)$$

Guidelines from the compendium of Fish and Fishery Products Processes, Hazards and Controls (Price and Tom, 2003) state that vacuum or vacuum modified atmosphere packaged smoked fish or smoked-flavored fish must contain not less than 3.5% water phase-salt in the loin muscle, or, where permitted, the combination of 3.0 % water phase-salt in the loin muscle and 100-200 ppm (parts per million) nitrite.

For air packaged smoked fish or smoked-flavored fish, not less than 2.5% water phase-salt in the loin muscle is required to prevent *Clostridium botulinum* growth and consequently toxin formation.

During the smoking process fish will be exposed to more than 350 chemical compounds produced by the smoke generators. The majority of these compounds are carbonylic and phenolic derivatives and their proportion will vary depending on the maximum temperature achieved in the generator (Guillen and Ibargoitia 1996).

The antimicrobial effect of the smoke is generally attributed to the combined action of formaldehyde, phenolic substances and acids (Suñen 1998; Thurette *et al* 1998; Niedziela *et al* 1998; Faith *et al* 1992; Messina *et al* 1988).

The above information provides ample evidence that *L. monocytogenes* contamination in ready-to-eat chilled VPCSS can occur at some stage during processing and, because this product supports *L. monocytogenes* growth during processing and storage, that new, non-thermal interventions or anti-listerial steps need to be introduced to minimize the risk of infection consequent to this contamination. In this Chapter are presented results of challenge studies involving three commercial antibacterial formulations against *L. monocytogenes* in deliberately contaminated cold smoked salmon. Special attention was given to the assessment of organoleptic changes that occur due to the addition of those treatments. Colour, texture and taste are crucial attributes for a successful cold smoked salmon and must be preserved as close as possible to the non-treated control sample.

MATERIAL AND METHODS

Anti-Listerial Treatments

The commercial treatments chosen in this experiment to challenge the growth of *Listeria monocytogenes* on cold smoked salmon were PURASAL Opti.Form4 at 1.5 and 3% w/w (Purac, liquid solution, 56% potassium lactate and 4% sodium diacetate, ratio 14:1 w/w), PURASAL Powder S 96 at 2.3% w/w (Purac, powder, 96% sodium lactate) and GUARDIAN NR100 at 0.1% w/w (Danisco, powder, 1.25% Nisin and min. 65% natural rosemary extract).

The dry antimicrobial treatments described above were applied during the dry curing step at the factory (*see* Fig. 1a.) whereas the liquid treatment was pipetted onto

the sliced cold smoked salmon, evenly spread with a sterile hockey stick and allowed to be absorbed for 10 minutes prior the *Listeria monocytogenes* inoculation and vacuum packaging which occurred in the microbiology laboratories at the University of Tasmania.

Cold smoked salmon sample preparation

Farmed raw Atlantic salmon (*Salmo salar*) were sourced and processed into VPCSS by Aquatas Ltd, a local smokehouse in Hobart, Tasmania. The process included filleting, dry salt curing, smoking, slicing, vacuum packing and refrigerated storage at 4°C and 10°C.

Organoleptic and microbiological samples from untreated (control) and treated samples were evaluated at the same time so both measurements could be later correlated. To minimize variations in fat content, smoke absorption, taste, colour and ultimately overall acceptance of the finished product both treated and untreated samples were obtained from the same fish (i.e., each fish yielded 2 fillets, 1 fillet was treated and the other was used as control).

A sterile stainless steel template of 4 x 4 cm (16 cm^2) was used to cut the cold smoked salmon slice into squares (*see* Fig. 1b). Because of the uniform slice thickness of 2 - 2.5 mm each square weighted approximately 5g. This weight was particularly important when calculating the amount of the anti-listerial liquid (w/w) to be used as per manufacturer's guidelines.

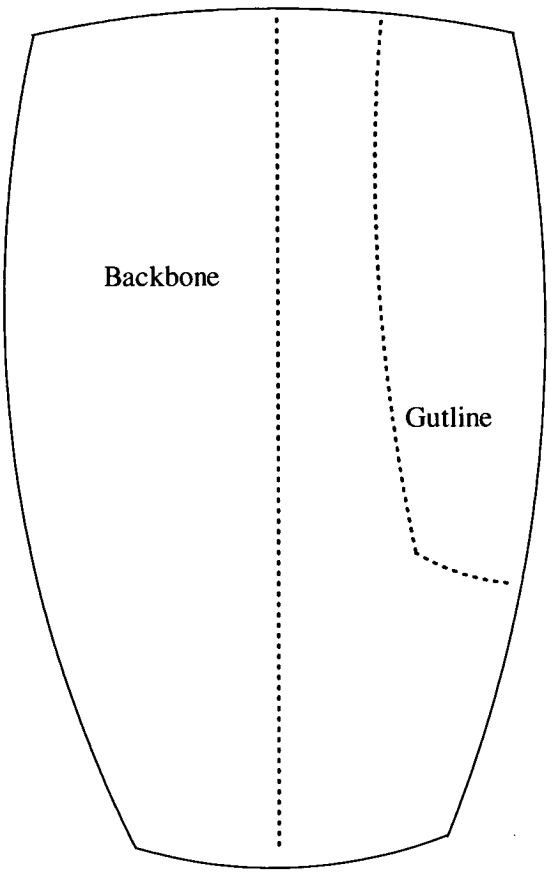


Fig. 1a. Cold smoked salmon **fillet** prior to slicing – view from the top

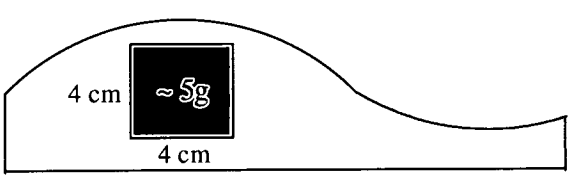


Fig. 1b. Cold smoked salmon **slice** – top view, showing the section taken for the inoculation challenge studies with *L. monocytogenes.*

Bacterial culture, inoculation and enumeration

Strain Scott A, the type strain of *Listeria monocytogenes* was used in all experiments and was provided by Food Science Australia[4], North Ryde, Sydney.

Cryovials containing the stock culture were maintained in 10% glycerol (vol/vol) at -20°C. Bacteria were grown overnight on Tryptone Soya Broth with 0.6% yeast extract (TSB-Ye) at 37°C. The logarithmic phase culture was diluted in peptone water (0.1%) with 0.85% NaCl and 100µL of a 5 x 10^3 cfu/ml suspension was evenly distributed to each of the 5g cold smoked salmon squares (16 cm^2). The aim was to inoculate samples with 10^2-10^3 *Listeria monocytogenes* cfu/g. Samples were then vacuum packed in a film with low gas permeability (Cryovac Tufflex LM plastic bags made of nylon/bond/LLDPE, 120 micron thickness, oxygen permeability of 55cc/m^2/24hrs/atm at 23 °C and 75% RH) and kept at 4°C and 10°C for up to 44 days. Aerobic plate count (APC) was also evaluated as a means to monitor the product's shelf-life as dictated by regulatory considerations discussed in Chapter 1, i.e. levels less than 10^5 cfu/g. This was done as a simple screen of change in cold smoked salmon shelf-life due to the treatments applied. Sample analyses were carried out in replicate at all times.

L. monocytogenes were enumerated on PALCAM (Oxoid, CM0877 with SR150 selective supplement) agar enriched with selective supplement after 48h incubation at 37°C. Nutrient Agar (NA, Oxoid CM0003) was used to enumerate the total aerobic count after 72h incubation at 20°C. For each treatment two replicate

[4] Food Science Australia is a joint venture between CSIRO and the Victorian Department of Primary Industries.

samples were analyzed at each sample time and the mean of results calculated and presented.

Sensorial Analysis

Standards Association of Australia (AS 2542.2.3, 1995) describes the use of Hedonic rating to measure the degree of liking of one or more of the sensory attributes of different samples. Testing conditions complied with standard AS 2542.1.

A seven-point hedonic scale was chosen for the preference testing of treated products. Ratings from each sample were given numerical values ranging from "Loved" (7) to "Hated" (1). The whole scale was: Loved (7), Extremely Liked (6), Liked (5), Neutral (4), Disliked (3), Extremely Disliked (2) and Hated (1).

The taste panel was composed of five trained employees of Aquatas and the attributes measured for both non-inoculated controls and treatments were colour, taste and overall acceptance. Sensorial analyses were only carried out on samples treated with PURASAL S96 and GUARDIAN NR100 because of their "powder" format and availability to the industry. As previously explained those treatments were carried out at Aquatas Ltd whereas the liquid treatment, PURASAL OptiForm4, was performed at the microbiology laboratory at University of Tasmania.

RESULTS AND DISCUSSION

Sodium and potassium lactate at levels varying from 2 to 4% have a bacteriostatic effect on microbial populations (i.e. they increase the lag phase and reduce the rate of growth of populations) and this has been directly shown for a number of pathogenic bacteria including *L. monocytogenes, E. coli* O157:H7 and *Salmonella* spp. (Pelroy *et al* 1994; Yoon *et al* 2004). The effectiveness of these

agents increase with increasing concentration and can be used in liquid form (60% w/w) or powder (up to 96% w/w). Liquid solutions are used more frequently because they can be mixed with brine and injected into the product. Both salts have been shown to decrease water activity and this adds to the inhibition of bacterial growth. Additionally, when in solution these salts dissociate, the ions react with water and ultimately form lactic acid. Organic acids are weak acids. As a weak acid, lactic acid has further antimicrobial activity in that it can cross the bacterial membrane in its undissociated form because it is more lipophilic than the dissociated form. Once inside the cell, the more neutral environment causes the lactic acid to dissociate, releasing a proton, and, thereby, reducing the pH of the cytoplasm. The cell will try to maintain its internal pH by neutralising or expelling protons but this slows the cell's growth, e.g. because energy is diverted from growth-related functions (Adams and Moss., 2007).

Figure 2 compares the growth of *L. monocytogenes* inoculated on VPCSS and left treated with Purasal 96S containing 96% w/w sodium lactate and stored at 4 and 10°C with samples that were not treated. When the powder was used at 2.34% w/w, *L. monocytogenes* growth in VPCSS was inhibited for 30 days when stored at 4°C. *L. monocytogenes* were able to grow in the presence of the antimicrobial when stored at 10°C but had a longer lag phase and slower growth rate. Therefore, while proper temperature control is crucial to inhibit the growth of *L. monocytogenes* in VPCSS, when stored at 4°C, the addition of sodium lactate could greatly reduce the risk of food poisoning by *L. monocytogenes* in that product.

The organoleptic trial results showed no statistically significant difference in the colour, taste and overall acceptance of untreated and treated samples in this study (*see* Table 4), although there was a slight preference for the treated samples.

This is in accordance with the organoleptic trial conducted by Simmons *et al* (2000) who considered that this was a consequence of the higher sodium content in the treated product. Table 4 below summarizes such sensorial analysis results. Further details of the experimental design and statistical evaluation of results can be found in Appendix 1.

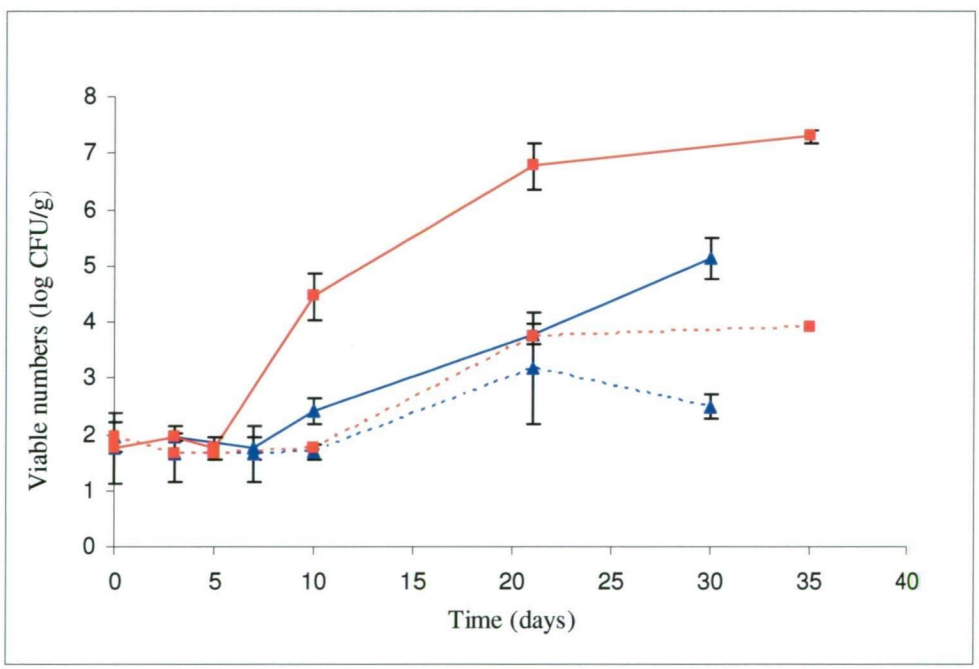


Figure 2 The growth of *L. monocytogenes* on vacuum-packed cold smoked salmon stored at 4 (blue) or 10°C (red). Samples were untreated (solid lines) or treated (dashed lines) with 2.34% w/w Purasal S96.

Table 4 Statistical sensorial analysis of sodium lactate treated samples

		Day Zero			Day 3			Day 7			Day 10			Day 21			Day 30		
Tasters	**Treatment**	C*	T*	O*	C	T	O	C	T	O	C	T	O	C	T	O	C	T	O
1	**Control**	NA	NA	NA	5	3	4	5	3	4	5	5	5	5	4	5	5	2	2
	Na-L	NA	NA	NA	5	5	5	5	5	5	5	5	5	5	5	5	5	2	3
2	**Control**	6	6	6	6	4	4	6	3	3	5	4	4	5	3	3	5	2	2
	Na-L	3	4	4	6	5	5	6	6	6	5	4	4	5	2	2	5	2	3
3	**Control**	5	4	4	6	3	4	5	3	3	5	4	4	5	3	4	5	2	2
	Na-L	5	4	4	5	5	5	4	4	4	5	5	5	3	3	3	5	2	2
4	**Control**	7	7	7	7	5	5	6	2	2	5	4	4	6	3	3	NA	NA	NA
	Na-L	5	3	3	7	5	5	4	4	4	5	6	5	5	3	3	NA	NA	NA
5	**Control**	5	6	6	5	4	4	5	4	4	5	3	4	5	3	4	5	4	4
	Na-L	5	5	5	5	5	5	5	5	5	5	5	5	5	4	4	5	2	2
Average	**Control**	5.8	5.8	5.8	5.8	3.8	4.2	5.4	3.0	3.2	5.0	4.0	4.2	5.2	3.2	3.8	5.0	2.5	2.5
	Na-L	4.5	4.0	4.0	5.6	5.0	5.0	4.8	4.8	4.8	5.0	5.0	4.8	4.6	3.4	3.4	5.0	2.0	2.5
StD	**Control**	1.0	1.3	1.3	0.8	0.8	0.4	0.5	0.7	0.8	0.0	0.7	0.4	0.4	0.4	0.8	0.0	1.0	1.0
	Na-L	1.0	0.8	0.8	0.9	0.0	0.0	0.8	0.8	0.8	0.0	0.7	0.4	0.9	1.1	1.1	0.0	0.0	0.6

* C = colour, T = taste, O = overall acceptance

p-values

Day	0	3	7	10	21	30
Colour	0.4525	0.0176	0.3239	0	0.3239	0
Taste	0.3942	0.1655	0.0461	0.2944	0.1799	0.5211
Overall	0.3942	0.1019	0.0677	0.1943	0.0207	0.8931

Treatment of VPCSS inoculated with *L. monocytogenes* with PURASAL Opti.Form4 inhibited the growth of *L. monocytogenes* at both 4 and 10°C when used at a concentration of 1.5% w/w. When the concentration of the antimicrobial liquid mixture was increased to 3% w/w, some listericidal properties were evident. Those results are shown in Figure 3.

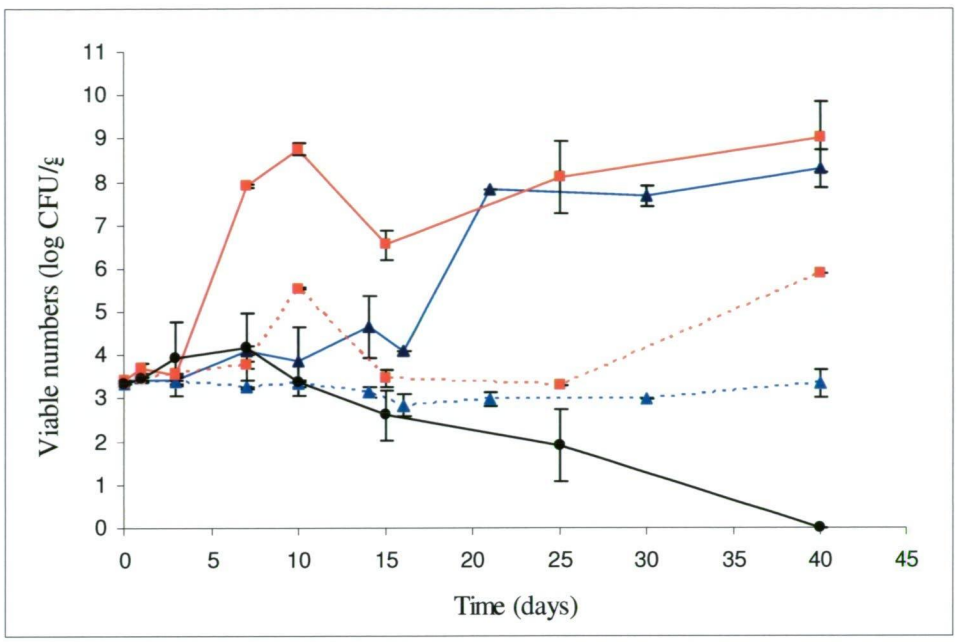


Figure 3 The growth of *L. monocytogenes* on vacuum-packed cold smoked salmon stored at 4 (blue) or 10°C (red). Samples were untreated (solid lines) or treated with 1.5% w/w PURASAL Opti.Form4 (dashed lines). The data in black show the listericidal effect of the same antimicrobial product when used at a concentration of 3% and when the fish product was stored at 10°C.

One drawback of PURASAL Opti.Form4 is its liquid nature. While it was effective in these experiments, it was applied to the surface of individual slices. It would be very difficult to achieve appropriate dispersion of it throughout the product during normal processing unless the manufacturer injects the fillets with the brine/treatment solution instead of applying it mixed with dry salt at the curing step. This could potentially create another problem, that is, brine contamination and possible bacterial inoculation inside of the once sterile flesh with contaminants from the fish surface or from the brine solution itself, especially if it is to be recycled.

A blend of Nisin and rosemary extract (GUARDIAN NR100) was applied to the salmon surface at the factory and allowed to penetrate for 20 minutes prior the application of the curing salts. The curing salts permeated throughout the salmon fillets but the green Nisin blend appeared to remain on the surface of the fillets. No growth inhibition was observed in the deep inoculated (i.e. after slicing) treated samples at 4 or 10°C, this also indicates the absence of the Nisin in the flesh of the treated samples. This is clearly evident as per the Figure 4, overleaf, as no significant difference can be seen between treated and untreated samples.

No sensorial difference was found by the panellists when comparing untreated and GUARDIAN NR100 treated samples probably because they were exactly the same as the compound did not permeate throughout the sample.

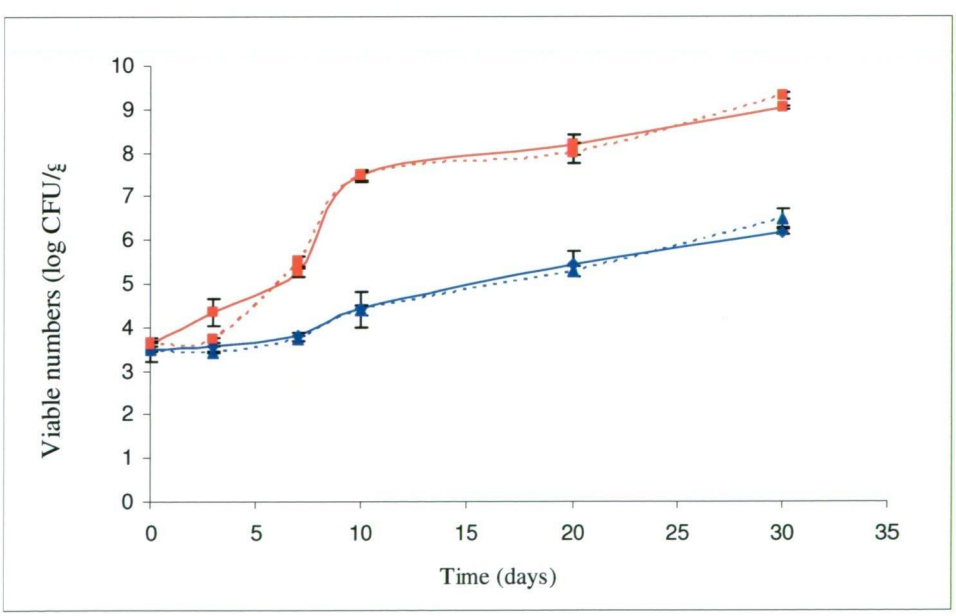


Figure 4 The growth of *L. monocytogenes* on vacuum-packed cold smoked salmon stored at 4 (blue) or 10°C (red). Samples were untreated (solid lines) or treated with 0.1% w/w Guardian NR100 (dashed lines).

Shelf-life evaluation

Shelf-life evaluation was initially based on total plate count (TPC) derived from nutrient agar plates incubated at 20°C for a period of 72h.

In practice, a commercial producer of CVPCSS needs to be able to achieve 28 days of "acceptable" quality to allow for distribution, retail display and sale, and to still provide sufficient high quality shelf life for consumers. TPC results (Figures 5 - 7) overleaf suggest that none of the treatments could be applied as shelf-life extenders under the studied conditions, if shelf life were assumed to end when TPC exceeds 5 x 10^5 cfu/g for a good quality product, as suggested by the International Commission for Microbiological Specifications for Foods for microbiological limits for fish and fishery products; Fresh and frozen fish and cold-smoked fish. (ICMSF, 1986 *cited in* http://seafood.ucdavis.edu/HACCP/Compendium/chapt09.htm).

Clearly, however, many CVPCSS products are already available on the market and, as suggested by the current results, microbiological levels do exceed the proposed regulatory limits. This highlights the irrelevance of the 5 x 10^5 cfu/g limit for high quality CSS product and ICMSF (*in press*) will move away from specification of microbiological limits as indicators of product quality (Ross, T., *pers. comm.*, 2009). As discussed in Chapter 1 there are many publications that highlight this issue, e.g. Leroi *et al* (2001) identified the need for more than one parameter (microbiological, physical or chemical) in order to provide a more reliable correlation between the shelf-life of cold smoked salmon than TPC alone can provide. They concluded that lactobacilli and yeast enumeration coupled with quantification of the total volatile

basic nitrogen (TVBN) are required to reliably determine the shelf-life of cold smoked salmon.

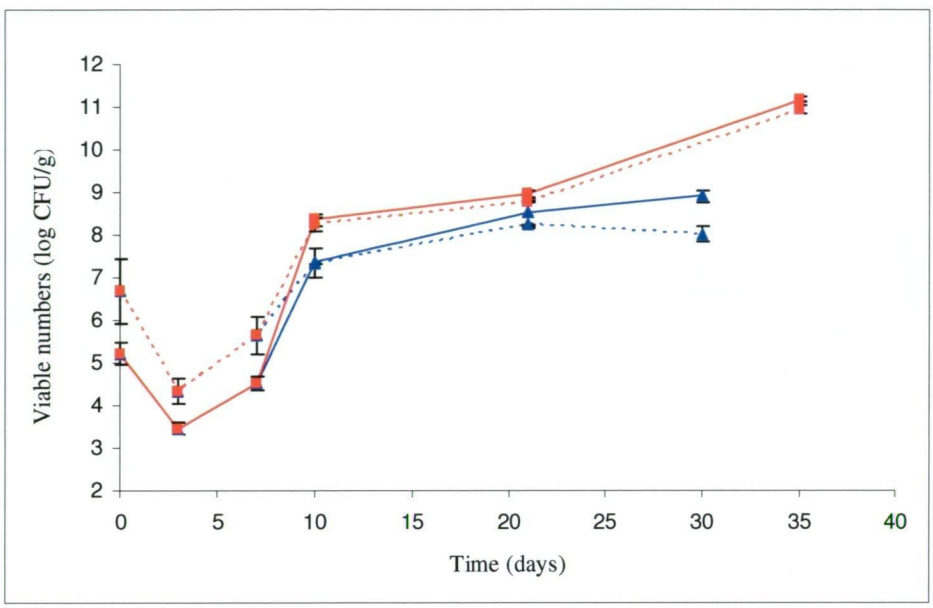


Figure 5 TPC on vacuum-packed cold smoked salmon stored at 4 (blue) or 10°C (red). Samples were untreated (solid lines) or treated with 2.34% w/w PURASAL S96 (dashed lines).

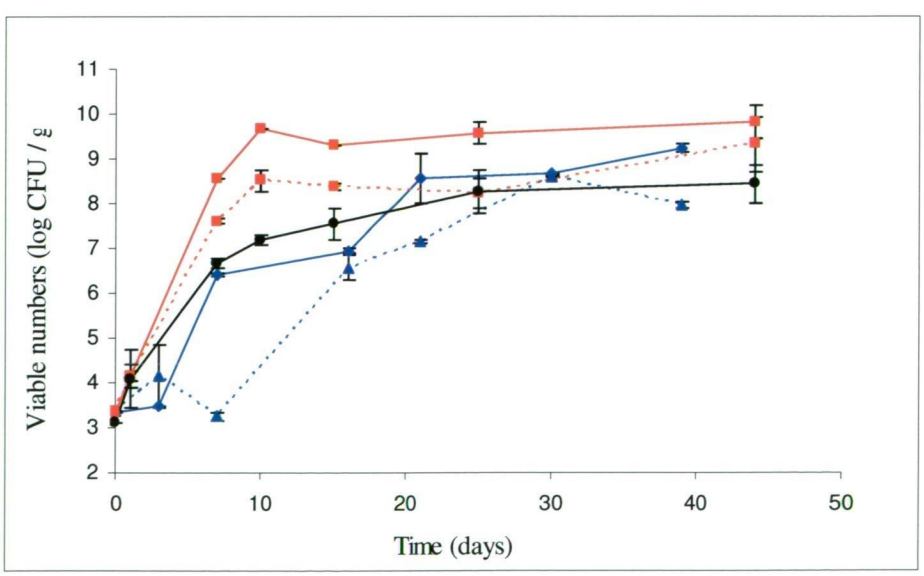


Figure 6 TPC on vacuum-packed cold smoked salmon stored at 4 (blue) or 10°C (red). Samples were untreated (solid lines) or treated with 1.5% w/w PURASAL Opti.Form4 (dashed lines). The data set in black shows the effect of the same antimicrobial product when used at a concentration of 3% and when the fish product was stored at 10°C.

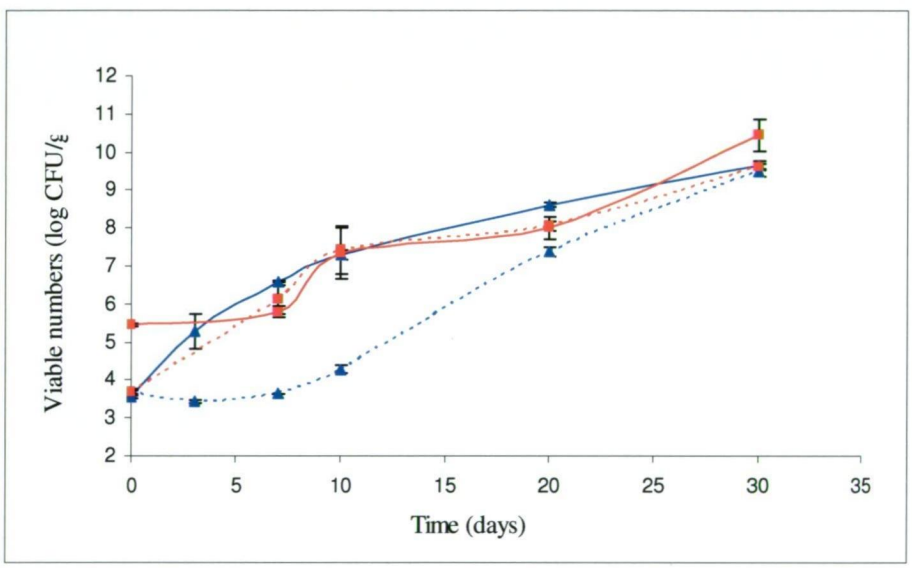


Figure 7 TPC on vacuum-packed cold smoked salmon stored at 4 (blue) or 10°C (red). Samples were untreated (solid lines) or treated with 0.1% w/w Guardian NR100 (dashed lines).

Guardian NR100 at 0.1% w/w was able to extend the lag phase of the treated samples but the bacteria were successful in recovering and achieved the same levels as the untreated samples over a period of 30 days. Shelf-life extension was once again unsuccessful.

CONCLUSIONS

- Sodium lactate powder and potassium lactate/sodium diacetate liquid mix inhibited *L. monocytogenes* growth and could be used as listeriostatic agents on CVPCSS that is intended to be kept at 4°C or below for up to 30 days,

- The same treatments described above also reduced the growth rate of *Listeria monocytogenes* at 10°C but the potassium lactate/sodium diacetate liquid mix seemed to be more effective than sodium lactate powder at this temperature,

- Under the experimental conditions assessed the Nisin/rosemary extract powder was unable to inhibit growth of *Listeria monocytogenes* inoculated onto slices, probably because it did not penetrate when initially applied to the salmon fillets,

- None of the treatments reduced the total microbial load to a level that could deliver an acceptable commercial shelf-life (i.e. $<5 \times 10^5$ cfu/g for 28 days at 4°C), that satisfies ICMSF (1986) recommended specifications,

- No significant difference ($p>0.05$) was noticed by the panelists when evaluating the colour, taste and overall acceptance of treated and non-treated samples, nor samples that exceed the above regulatory limits, reinforcing published studies that report that product quality of CSS is not well correlated to total plate count , and

- On the basis of its efficacy, relative ease of application, and absence of organoleptic changes, sodium lactate powder appears to be a viable and practical antilisterial treatment for CVPCSS.

ACKNOWLEDGMENTS

- Food Science Australia and the Australian Postgraduate Award scheme (DEST) for scholarship support,
- Dr Lisa Szabo and Dr Tom Lewis,
- Professors June Olley, David Ratkowsky and Tom McMeekin and Dr Lyndal Mellefont,
- Phil Walsh and colleagues at Aquatas - Tasmania,
- Ellis Cox, Stephen Ridge, Aung Zaw, Mark Jackson, Nazri Mohamed Naim, Pragash Devendran,
- Danisco and PURAC for provision of antilisterial compounds.

REFERENCES

Adams, M.R. and Moss, M.O. 2007. *Food Microbiology*, 3rd Edition. Published by Royal Society of Chemistry. Chapter 3, page 27.

AS 2542.0-1995. Australian Standard. Sensory analysis of foods. Part 0: Introduction and list of methods. Published by Standards Australia. NSW, 1995.

Autio, T., Hielm, S., Miettinen, A., Sjöberg, M., Aarnisalo, K., Björkroth, J., Mattila-Sandholm, T. and Korkeala, H. 1999. Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout plant detected by pulsed-field gel electrophoresis typing. *Applied and Environmental Microbiology* **65**:150-155.

Barakat, R.K. and Harris, L.J. 1999. Growth of *Listeria monocytogenes* and *Yersinia enterolitica* on cooked modified-atmosphere-packaged poultry in the presence and absence of a naturally occurring microbiota. *Applied and Environmental Microbiology* **65**:342-345.

Ben Embarek, P.K. 1994. Presence, detection and growth of *Listeria monocytogenes* in seafoods: a review. *International Journal of Food Microbiology* **23**:17–34.

Dalgard, P., and L.V. Jørgensen. 1998. Predicted and observed growth of *Listeria monocytogenes* in seafood challenge tests and in naturally contaminated cold-smoked salmon. *International Journal of Food Microbiology* **40**:105-115.

Dillon, R., Patel, T. and Ratman, S. 1994. Occurrence of *Listeria* in hot and smoked salmon seafood products. *International Journal of Food Microbiology* **22**:73-77.

Eklund, M.W., Poysky, F.T., Paranjpye, R.N., Lashbrook, L.C., Peterson, M.E. and Pelroy, G.A. 1995. Incidence and sources of *Listeria monocytogenes* in cold smoked salmon fishery products and processing plants. *Journal of Food Protection* **58**:502-508.

European Parliament and Council 1995. Directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners. Official Journal of the European Communities 38, 24.

Farber, J.M. 1991. *Listeria monocytogenes* in fish products. *Journal of Food Protection* **54**:922-924.

Faith, N.G., Yousef, A.E. and Luchansky, J.B. 1992. Inhibition of *Listeria monocytogenes* by liquid smoke and isougenol, a phenolic component found in smoke. *Journal of Food Safety* **12**:303-314.

FAO/WHO (Food and Agriculture Organization of the United Nations and World Health Organization). 2004. Risk assessment of *Listeria monocytogenes* in ready-to-eat foods. TECHNICAL REPORT. Microbiological risk assessment series 5. Food and 685 Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00100 Rome, Italy or http://www.fao.org/es/esn/food/risk_mra_listeria_report_en.stm

FDA/ORA (US Food and Drug Administration/US Office of Regulatory Affairs). 1996. Tuna, Sable, Salmon, Shad – smoked cured, adulteration involving food additives, sodium nitrite. Compliance Policy Guides Manual Sec. 540.500.

Guillen, M.D. and Ibargoitia, M.L. 1996. Relationship between the maximum temperature reached in the smoke generation processes from *Vitis vinifera* L. shoot sawdust and composition of the aqueous smoke flavouring preparations obtained. Journal of Agriculture and *Food Chemistry* **44:**1302-1307

Hilderbrand, K.S. 1991. Quick determination of water phase salt content of cold smoked fish. Oregon State University, Extension Sea Grant, Special Report 883, August.

Hudson, J.A., Mott, S.J., Delacy, K.M. and Edridge, A.L. 1992. Incidence and coincidence of *Listeria* spp., motile aeromonads and *Yersinia enterolitica* on ready-to-eat fleshfoods. *International Journal of Food Microbiology* **16**:99-108.

Huss, H. H, Embarek, P. K. B.and Jeppesen, V. F. 1995. Control of biological hazards in cold-smoked salmon production. *Food Control* **6:**335-340.

ICMSF (International Commission on Microbiological Specifications for Foods). 1986. *Microorganisms in Foods 2, Sampling For Microbiological Analysis: Principles and Specific Applications*, 2nd ed. University of Toronto Press, Buffalo, NY.

Jørgensen, L.V. and Huss, H.H. 1998. Prevalence and growth of *Listeria monocytogenes* in naturally contaminated seafood. *International Journal of Food Microbiology* **42**:127-131.

Jørgensen, L.V., Dalgaard, P. and Huss, H.H. 2000. Multiple compound quality index for cold-smoked salmon (*Salmo salar*) developed by multivariate regression of biogenic amines and pH. *Journal of Agricultural and Food Chemistry* **48**:2448-2453.

Leroi, F., Joffraud, J.J., Chevalier, F. and Cardinal, M. 2001. Research of quality indices for cold-smoked salmon using a stepwise multiple regression of microbiological counts and physico-chemical parameters. *Journal of Applied Microbiology* **90**:578-587.

Kanuganti, S.R., Wesley, I.V., Reddy, P.G., Mckean, J. and Hurd, H.S. 2002. Detection of *Listeria monocytogenes* in pigs and pork. *Journal of Food Protection* **65**:1470-1474.

Leroi, F., Joffraud, J.J., Chevallier, F. and Cardinal, M. 1998. Study of the microbial ecology of cold smoked salmon during storage at 8°C. *International Journal of Food Microbiology* **39**:111-121.

Leroi, F., Joffraud, J.J. and Chevallier, F. 2000. Effect of salt and smoke on the microbiological quality of cold smoked salmon during storage at 5°C as estimated by the factorial design method. *Journal of Food Protection* **63**:502-508.

Leroi, F. and Joffraud, J.J. 2000. Salt and smoke simultaneously affect chemical and sensory quality of cold smoked salmon during 5°C storage. *Journal of Food Protection* **63**:1222-1227.

McLauchlin, J. 1997. The pathogenicity of *Listeria monocytogenes*: a public health perspective. *Reviews in Medical Microbiology* **8**:1-14.

Messina, M.C., Ahmad, H.A., Marcello, J.A., Gerba, C.P. and Paquette, M.W. 1988. The effect of liquid smoke on *Listeria monocytogenes*. *Journal of Food Protection* **51**:629-631.

Niedziela, J.C., Macrae, M., Ogden, I.D. and Nesvadba, P. 1998. Control of *Listeria monocytogenes* in salmon: antimicrobial effect of salting, smoking and specific smoke compounds. *Lebensmittel Wissenschaft und Technologie* **31**:155-161.

Pelroy, G., Peterson, M., Paranjpye, R., Almond, J. and Eklund, M. 1994. Inhibition of *Listeria monocytogenes* in cold-process (smoked) salmon by sodium nitrite and packaging material. *Journal of Food Protection* **57**:114-119.

Price, R.J. and Tom, P.D. 2003. Compendium of fish and fishery product processes, hazards and controls, (http://seafood.ucdavis.edu/HACCP/compendium/compend.htm). Food Science and Technology, University of California (Davis).

Rørå, A. M. B., Kvale, A., Morkore, T., Rorvik, K.-A., Steien S. H. & Thomassen, M. S. 1998. Process yield, colour and sensory quality of smoked Atlantic Salmon in relation to raw material characteristics. *Food Research international* **31**:601-609.

Rørvik, L.M., Yndestad, M. And Skjerve, E. 1991. Growth of *Listeria monocytogenes* in vacuum packed smoked salmon during storage at 4°C. *International Journal of Food Microbiology* **14**:111-118.

Rørvik, L.M, Caugant, D.A. and Yndestad, M. 1995. Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. *International Journal of Food Microbiology* **25**:19-27.

Simmons, P.D., Otwell, W.S. and Rodrick, G.E. 2000. The effects of sodium lactate on cold smoked salmon. Department of Food Science & Human Nutrition, University of Florida, internet source (http://sst.ifas.ufl.edu/25thAnn/file03.pdf)

Suňen, E. 1998. Minimum inhibitory concentration of wood smoke extracts against spoilage and pathogenic micro-organisms associated with foods. *Letters in Applied Microbiology* **27**:45-48.

Truesltrup Hansen, L. 1995. Quality of chilled vacuum packed cold smoked salmon. *PhD thesis*. Royal Veterinary and Agricultural University, Frederiksberg, Denmark.

Truelstrup Hansen, L., Røntved, S.D. and Huss, H.H. 1998. Microbiological quality and shelf-life of cold-smoked salmon from three different processing plants. *Food Microbiology* **15**: 137-150.

Thurette, J., Membre, J.M., Ching, L.H., Tailliez, R. and Catteau, M. 1998. Behavior of *Listeria* spp. in smoked fish products affected by liquid smoke, NaCl concentration, and temperature. *Journal of Food Protection* **61**:1475-1479.

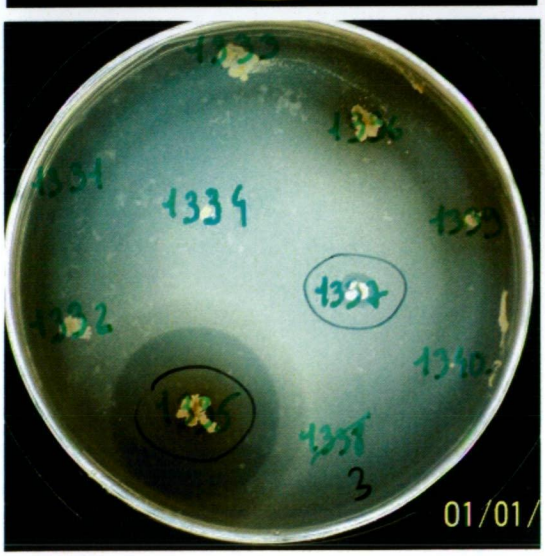
USDA/FDA (United States Department of Agriculture, and United States Food and Drug Administration). 2001. Draft Assessment of the relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat

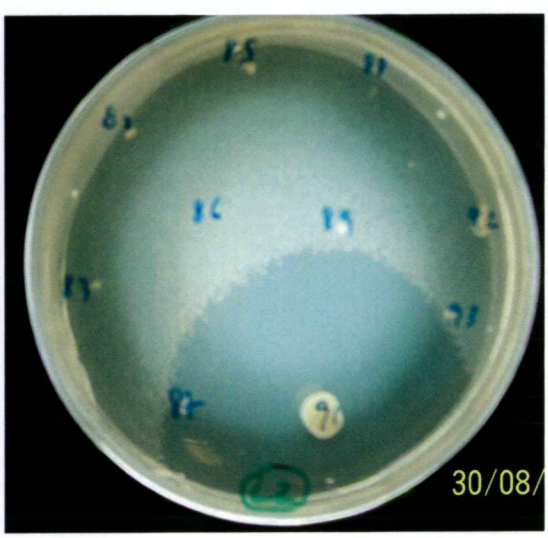
foods. Center for Food Safety and Applied Nutrition (FDA) and Food Safety Inspection Service (USDA) (Available at: www.foodsafety.gov/~dms/lmrisk.html).

Valenti, M., Daubert, S. Bucellati, L. and Cantoni, C. 1991. *Listeria* spp. in salmons. *Industrie Alimentari* **30**:741-743.

Yoon, K.S., Burnette, C.N., Abou-Zeid, K.A. and Whiting, R.C. 2004. Control of growth and survival of *Listeria monocytogenes* on smoked salmon by combined potassium lactate and sodium diacetate and freezing stress during refrigeration and frozen storage. *Journal of Food Protection* **67**:2465-2471.

Chapter 3: Screening Antarctic and Sub-Antarctic Soil-borne Bacteria for Potentially Novel Listericidal Bacteriocins





INTRODUCTION

The need for novel antibacterial compounds, and not just variations of the existent ones, is clearly evident as more and more new "superbugs" are able to develop resistance to currently available antibiotics. Emerging pathogens and the ever growing threat of biological warfare are additional serious reasons to pursue the search for such compounds (Raloff, 1998). This can be a very time consuming and expensive exercise estimated by DiMasi *et al* (2003) to be in the order of US$ 800 million from discovery to market. The difficulties described above are the main drivers for the poor discovery and approval of new antibacterial agents. Since 1962 only three new classes of antibacterial agents, including oxazolidinones, cyclic lipopeptides and pleuromutilin derivatives, have been approved for clinical use (Leeb 2004; Jacobs 2007).

According to Klaenhammer (1988) nearly all bacteria produce antimicrobial compounds called bacteriocins. Bacteriocins are antimicrobial peptides and are thought to act as a defense system against other closely related bacteria which may be competitors.

When investigating novel bioactive compounds, including compounds capable of being active and/or stable under extreme conditions it is logical to think that the environment where they co-exist has to be extreme, or unusual, as well. Antarctica is one of the most extreme environments on earth with its wide range of temperature variations, high ultraviolet radiation levels and low water availability, being the driest continent on Earth.

With that in mind the University of Tasmania in partnership with an Australian private biotechnology company (Cerylid Pty., Ltd.) investigated the potential of the soil-borne Antarctic bacteria to produce an antibacterial compound or elucidate the mechanism that offers protection to them against the exposure to the high UV levels commonly experience in Antarctica. With this collaborative project finishing, this collection of Antarctic Actinobacteria was available for investigation and provided an unique opportunity to access the antimicrobial potential of these strains.

In this study the potential of organisms in the above culture collection to inhibit the growth of five different *Listeria monocytogenes* strains, including the type strain Scott A, was investigated. This study also included the partial characterisation of the compounds of interest.

This screen was carried out in collaboration with Mr. Jimmy Twin, a PhD student examining the taxonomy of Antarctic bacteria, with an interest in discovering novel microbiota from this collection of Actinobacteria, and so provided useful information regarding the molecular identification and phylogeny of the antimicrobial producing strains gleamed from this study. As the primary investigator in this study I carried out the majority of the screening work as well as the initial characterization of the compounds produced by the Actinobacteria while Mr. Twin undertook the molecular identification and creation of the phylogenetic tree as well as assisting with some of the screening activities.

Specificity is a characteristic of bacteriocins and in comparison to antibiotics; they possess a relatively narrow bactericidal spectrum and are thought to be toxic to bacteria closely related to the species of the bacteriocin producer (Riley and Wertz, 2002).

O'Brien *et al* (2004) suggested that Antarctic bacteria produce cold active antimicrobial compounds. Their challenge bacteria were from different genera and species, but not strains. The studies described in this Chapter focused on a single target bacterial species (*Listeria monocytogenes*) with activity against multiple strains assessed. The goal was to discover a bacteriocin with broad listericidal capacity.

MATERIAL AND METHODS

Bacterial source and strains

Approximately 1600 of soil-borne bacteria belonging to the Actinobacteria phylum were screened against five different *L.monocytogenes* strains for their ability to produce bacteriostatic/bacteriocidal compounds.

The Actinobacteria were isolated from either Antarctic or sub-Antarctic regions, primarily from the Vestfold Hills region, including Davis Station (68°S, 78°E), and from Macquarie Island (54°S, 158°E). The collection had been morphologically identified as being Actinobacteria, primarily of the genus *Streptomyces* (approx. 75% of strains). The original isolate numbers ranged from A10000004 to A10001700 but, for convenience, are referred to as strains 4 to 1700 only (it is important to keep the reference numbers for future researchers when referring to this collection).

Vials containing the 1600 isolates were kept at all times in 30% v/v glycerol stocks at -80°C. Food Science Australia (North Ryde, Sydney, NSW), an arm of the Commonwealth Scientific and Industrial Research Organisation, provided 4 of the 5 *L. monocytogenes* strains used in this challenge study. They were FRRW 2343 (isolated from salad with pasta, cheese & ham/bacon), FRRW 2345 (isolated from ham), FRRB

2472 (Scott A) and FRRB 2542 (isolated from salami). The remaining strain, L5-22, is part of the University of Tasmania, School of Agricultural Science culture collection and was originally isolated from cold smoked salmon. For this experiment they will be referred as L1 (FRRW 2343), L2 (FRRW 2345), L3 (FRRB 2472), L4 (FRRB 2542) and L5 (L5-22).

Culture conditions and screening method

A similar screening method to the one used by O'Brien *et al* (2004) based on agar diffusion was chosen. Briefly, after cultures had been checked for purity on tryptone soya agar with 0.6% yeast extract (TSA-Ye[6], Oxoid CM0989) the Actinobacteria were stab inoculated on Marine Agar (MA[5]) plates and incubated at 10°C for 14 days to ensure colony formation. The Petri dishes (90 mm) were then overlayed with 20-25 ml of soft (0.75%) TSA-Ye containing one of the five *L. monocytogenes* strains at a concentration of approximately 10^6 cfu/ml. Plates were further incubated at 10°C for 7 days to allow for *L. monocytogenes* growth and allow formation of zones of clearance around actinobacterial colonies. If the zone of clearing was at least 2 mm from the outer edge of the Actinobacteria colony to the end of the clearance zone it was considered to be a positive result against the target strain. The entire screening process was carried out in duplicate.

Figures 1 and 2, overleaf, summarize the agar diffusion method described above.

5 Marine agar (MA): 5g peptone + 1g yeast extract + 15g Agar + 35g Sea salts

6 TSA-Ye: 6g yeast extract + 15g Agar + 33g tryptone soya broth (Oxoid CM 0989)

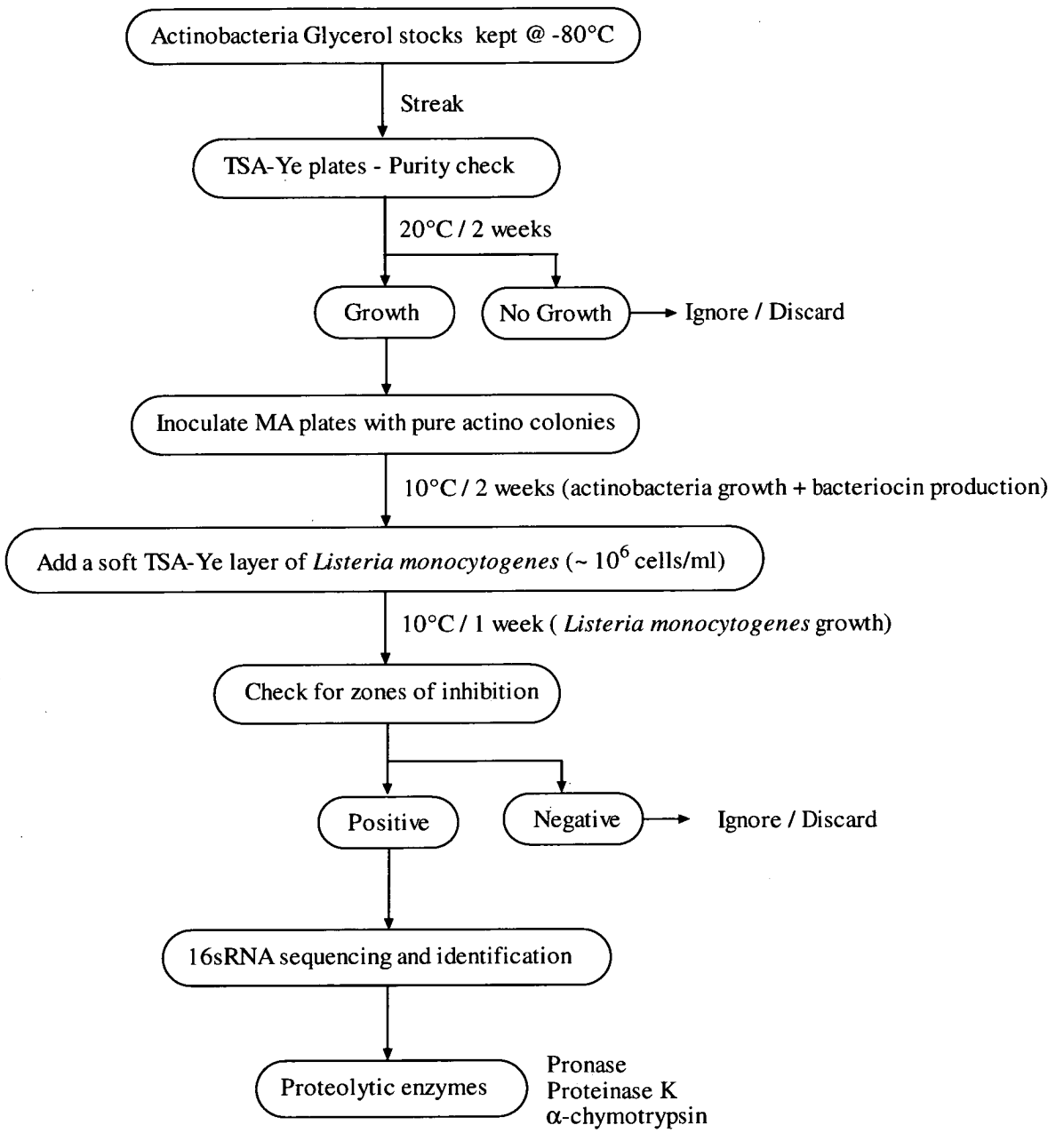


Figure 1 Flow Chart of Screening Method for Identification of Antarctic Actinobacteria that Produce Anti-listeiral Compounds

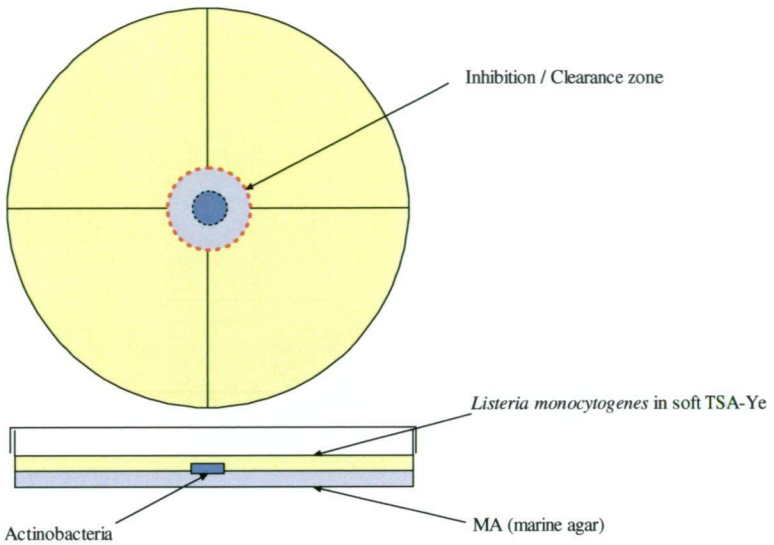


Figure 2 MA / TSA-Ye sandwich layer in a Petri dish illustrating an Actinobacteria colony and zone of inhibition

Preliminary characterization of the bacteriocin

To verify whether any anti-listerial compounds produced were of a proteinaceous nature, (indicative of a bacteriocin or antimicrobial peptide), the proteolytic enzymes pronase, proteinase K and α-chymotrypsin (Sigma Aldrich) were applied during the agar diffusion assays. The method was developed "in-house" based on analogous published studies. In summary, plates inoculated with Actinomycetes were incubated at 10°C for 14 days on MA before 5 µL of a 25 mg/ml solution of each of the enzymes above were injected around the colony at a 25mm distance from the outer edge of the colony. Plates were incubated at 37°C for 1 hour. Enzyme treated plates were then overlayed with a soft TSA-Ye lawn containing approximately 10^6 *L. monocytogenes* cells/ml and were then incubated at 10°C for 14 days.

Molecular Identification of Isolates

The following methodology for identification of isolates is reproduced from Twin (2008).

DNA extraction of bacterial colonies

A pure bacterial colony from an agar plate was placed in a 1.5mL screw capped tube containing 0.1mm, 0.5mm, and 1.0mm silicon-zirconium beads (Daintree Scientific) in 500µL saline EDTA. This cell suspension was frozen using liquid nitrogen, followed by shaking the tubes in a bead-beating device using 0.1 mm zirconium-silica sand for 10 seconds at 5000rpm to disrupt colonies.

This was repeated to ensure all cell mixtures had completely thawed. Cellular debris and beads were pelleted via centrifugation at 10000xg for 5 minutes in a bench top centrifuge with the upper supernatant undergoing two chloroform:isoamyl alcohol (24:1) washes. The resulting DNA-containing fraction then underwent purification using the UltraClean 15 DNA Purification Kit (MO BIO Laboratories, Inc., Cat #12100-300) following manufacturer's instructions.

PCR amplification of 16S rRNA gene

The 16S rRNA gene sequences of the DNA extracted were PCR amplified as follows. Briefly, 5µL of template DNA was added to 45µL of a master mix which consisted of 25µL of HotStarTaq Master Mix (Qiagen Pty Ltd Cat. # 203445), 5pmol of each primer, making up to the required volume with Qiagen supplied dH_2O. The HotStarTaq Master Mix contains 2.5 units of HotStarTaq DNA Polymerase, buffer containing 1.5mM $MgCl_2$, and 200µM of each dNTP. The primers used were the universal bacterial primers 10f (5'-GAGTTTGATCCTGGCTCAG-3') and either 1492r (5'-TACGGYTACCTTGTTACGACTT-3') or

1520r (5'-AGAAAGGAGGTGATCCAGCC-3') (Lane 1991). Amplicons were generated using a MJ Research PTC-200 peltier thermal cycler using the following program: one cycle of 15 minutes at 95°C; 30 cycles of 1 minute at 94°C, 1 minute at 52°C, 1.5 minute at 72°C; and a final extension step of 10 minutes at 72°C. The resulting PCR product was then purified using a QIAquick Spin column (Qiagen Pty Ltd Cat. # 28106), and visualised on a 1.5% agarose gel stained with 500ng/ml Ethidium bromide.

DNA sequencing of PCR products

DNA sequences were obtained from purified PCR products using a CEQ™ 8000 Genetic Analysis System (Beckman Coulter Inc). The sequencing reaction itself was a modified version of the protocol recommended with the GenomeLab DTCS Quick Start Kit (Beckman Coulter Inc., Cat #608120). The modification occurred with the set up of the sequencing mix that consisted of 4µL DTCS Quickstart mix, 2µL of primer at a concentration of 2pmol/µL, 10µL sterile milliQ H_2O, plus 4µL of purified PCR product. The primers used for sequencing comprised of the original primers described above plus 519f (5'-GWATTACCGCGGGKGCTG-3'), and 907r (5'-CCGTCAATTCCTTTGAGTTT-3') (Giovannoni,1991). The creation of labelled product was carried out on a MJ Research PTC-200 peltier thermal cycler using the following program: 30 cycles of 20 seconds at 96°C, 20 seconds at 50°C, and 4 minutes at 60°C. Prior to being sequenced the labelled PCR products were desalted using 100% and 70% ethanol washes as recommended by Beckman Coulter Inc.

Sequence analyses

Raw sequence files (*.scf) were exported to the Sequencher 4.5 program (Gene Codes Corporation) where chromatograms were analysed and sequence fragments were

aligned. Consensus sequences were then compared against other sequences on the Genbank database (Benson *et al* 2003) using the BLASTN function (Altschul *et al* 1990). The closest matches of each clone, as well as the closest cultured match were imported into the program Seaview (Galtier *et al* 1996), where all sequences were aligned using the ClustalW function. Neighbor joining trees calculated using the Kimura 2 parameter model were then constructed using the program Phylowin (Galtier *et al* 1996) with the resulting tree output made into a publishable quality using Adobe Photoshop CS 8.0 or Adobe Illustrator CS3 13.0.0 (Adobe Systems Inc.).

RESULTS

Growth and bacteriocin production rate

The initial number of 1600 isolates was reduced to 1263 because 21.1% (337) failed to recover from the glycerol stocks during the first incubation at 10 °C for 14 days on MA. From the remaining 1263 isolates, 199, a positive rate of approximately 16%, were able to inhibit the growth of one or more of the five *Listeria monocytogenes* strains evaluated. Detailed results of the screening process are presented in Appendix 1.

The positive 199 Actinomycetes were again screened in duplicate against the five challenge strains. This second screen identified 20 isolates capable of inhibiting all five *Listeria monocytogenes* strains with clearance zones larger than the 2mm benchmark previously set as a minimum. Photographs of culture plates from the screening process are presented in Appendix 2. Tables 1 and 2 below summarize the results from the second screen.

Table 1 Number of Actinobacteria isolates that inhibited any or all of the *Listeria monocytogenes* strains

L. monocytogenes **strain**	**No. Actinomyctes that inhibit Growth of *L. monocytogenes* strain**	**% of 199**
L1	26	13
L2	52	26
L3	49	25
L4	58	29
L5	42	21
All 5 strains	20	10

Identification of the bacteriocin producers

Twelve of the 20 isolates presented different sequences, strains 1087, 1065, 1204 and 1366 sharing 100% similarity as did strains 1139 and 1120; 1174 and 1138; 515 and 1478; and 1496 and 91. All strains belonged to the genus *Streptomyces.*

The phylogenetic relationships of these strains with other *Streptomyces* spp. are shown in Figure 3. The isolate showing the least amount of similarity to any described *Streptomyces* species is 1477, being 98.9% similar to *S. sanglieri* over a 1363bp region of their respective 16S rRNA gene sequences. It also shares a 99.6% match with *Streptomyces sp.* YIM26, an uncharacterised psychrotolerant *Streptomyces sp.* isolated from soil in China, over a 1406bp region.

Table 2 Zone of inhibition produced by the 20 Actinobacteria against *L. monoctogenes*

	Isolate no.	Zone of Inhibition (mm) produced
1	91	25
2	199	15
3	481	5
4	515	10
5	1065	5
6	1087	5
7	1120	5
8	1138	15
9	1139	5
10	1174	10
11	1202	20
12	1204	5
13	1257	5
14	1335	5
15	1366	15
16	1419	20
17	1477	20
18	1478	5
19	1496	20
20	1525	5

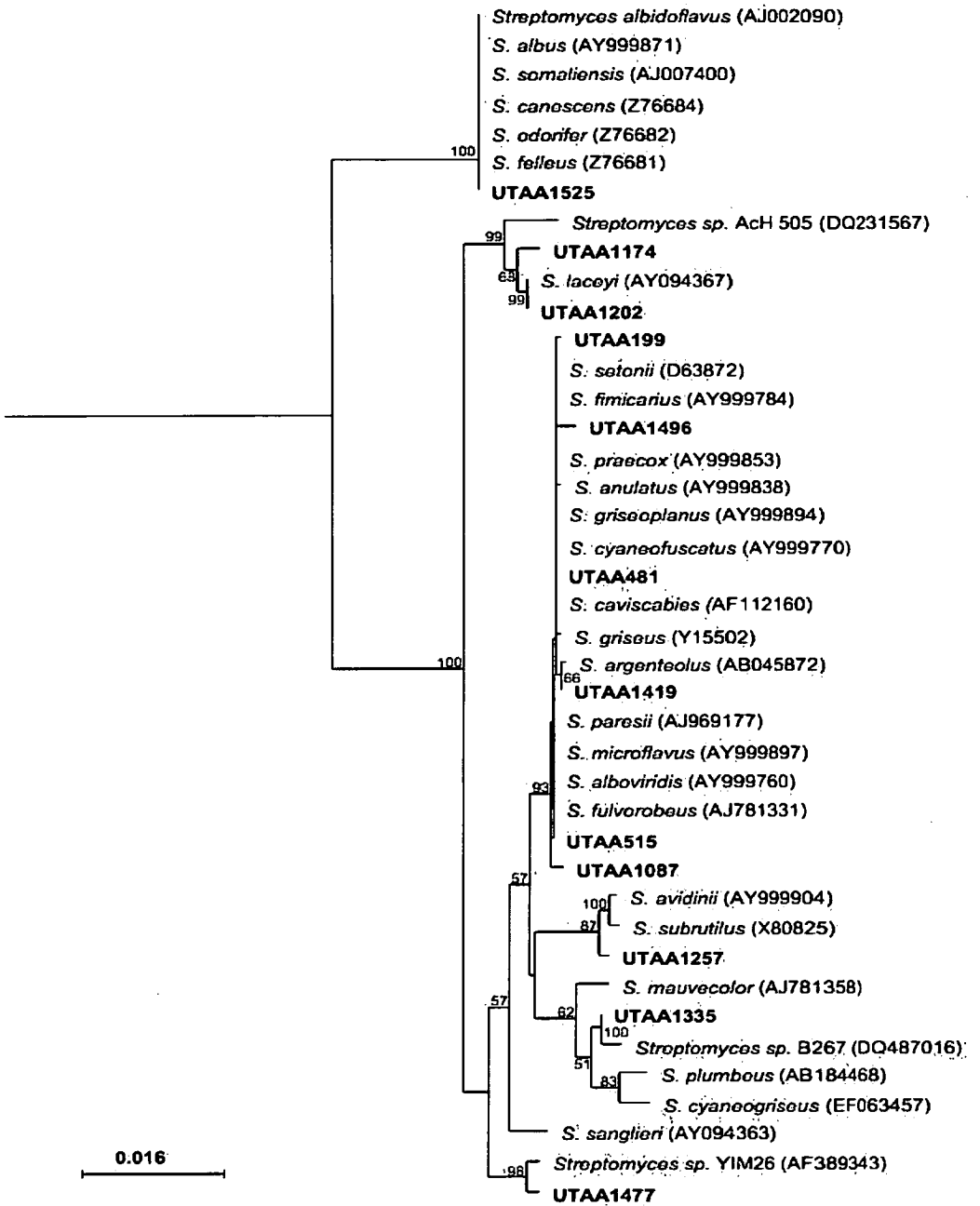


Figure 3 16S rRNA gene Neighbour Joining tree of *Streptomyces* isolates with closest matches from Genbank based on a total alignment length of 1317bp. *Bifidobacterium bifidum* (AY694148) and *Arthrobacter globiformis* (X80736) were used as outgroups, and comparisons were made using the Kimura two-parameter model with 1000 bootstrap replicates. Only bootstrap values greater than 50 are shown. The scale bar indicates 0.014 changes per nucleotide. (Twin, J., *personal communication*, 2009).

Bacteriocin's protease sensitivity

The degradation or complete negation of the clearance zone in the vicinity of the colony of the bacteriocin producer in the presence of any one of the proteases was considered a positive response. In other words, the enzyme was effective in disrupting or inactivating the antimicrobial produced allowing the challenging *Listeria monocytogenes* strain to grow around it.

Four of the 20 antimicrobials discovered appeared to be of proteinaceous nature. The α-chymotrypsin enzyme showed a positive reaction against the isolates 199, 481, 1138 and 1496. Pronase and proteinase K were effective against the antimicrobials produced by the isolates 199, 481 and 1496.

Figure 4 shows a partial degradation of the antimicrobial produced by the isolate 199 when exposed to the α-chymotrypsin.

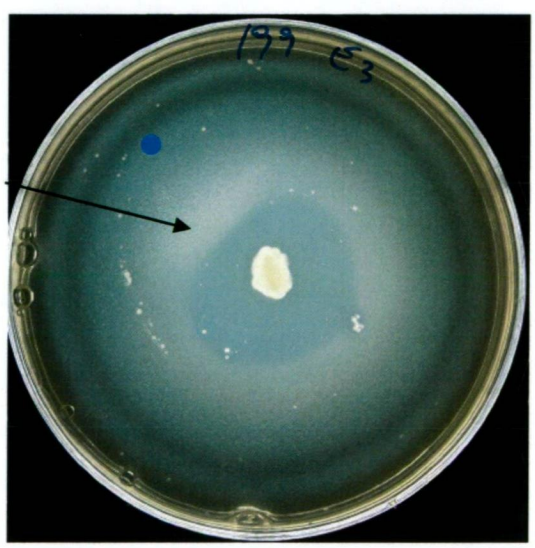


Figure 4 Partial enzymatic antimicrobial degradation caused by α-chymotrypsin (E3). The blue dot marks where the α-chymotrypsin solution was applied. The zone between the blue dot and the isolate 199 colony (white) shows a zone of negation (degradation of the antimicrobial) indicated by the arrow.

DISCUSSION

The discovery, characterization and commercialization of natural compounds that are active in cold environments, i.e. the temperatures experienced by perishable food products, would be of great value to the food industry. They could specifically target pathogens such as *Listeria monocytogenes* or another cold adapted microorganism capable of growing at refrigerated temperatures. Bacteriocins or bacteriocin producing bacteria, also known as protective cultures (PCs), can be added to food to increase the shelf-life or safety of such products.

The current technological and practical factors limiting the use of PCs, include but are not limited to, the effects of PCs on the sensory properties of the food in question; heat sensitivity of the PCs; inoculum size and lack of data on interaction between the PCs and FPSOs (food poisoning or spoilage organisms) (Rodgers *et al* 2002), and need to evaluate potential effects on consumers. Those limitations enhance the application of the purified bacteriocin instead of the bacteriocin-producing bacteria to the food to be preserved.

Bacteriocin producers and characteristics

Streptomyces, the main genus in the Antarctic Actinobacteria collection analysed, are typically aerobic soil-borne, spore forming Gram-positive bacteria with a high G+C content (>70%). Their ability to thrive in low nutrient environments and play an important role in decomposition and recycling of biomaterials is well known. They are also renowned for their ability to produce bacteriocins that are ribosomally synthesised peptide antibiotics (Jack *et al* 1995). This is a particular characteristic of bacteriocins produced by Gram-positive bacteria. In fact, *Streptomyces* are the largest antibiotic-producing genus in the microbial world (Berdy 2005) and their ability to synthesize

secondary metabolites for human medicine, agriculture and industrial applications is unique and well documented. Gram-positive bacteria produce smaller (3-6 KDa) bacteriocins than the ones produced by Gram-negative and can be divided in two large classes: lantibiotics and non-lantibiotics. Lantibiotics are further subdivided in class I (type A and type B), class II (a, b and c) and class III. The lantibiotics are small peptides (<5 KDa) containing unusual amino acids including lanthionine, α-methyllanthionine, dehydroalanine and dehydrobutyrine (Chen and Hoover 2003; McAuliffe *et al* 2001).

Currently none of the bacteriocins discussed in Chapter 1, section Bacteriocins and Bacteriocin Producers, are approved to be used in seafood products in Australia (Szabo *et al* 1999). The regulatory approval for those products can be a long and costly process but the return would certainly be very attractive.

This work demonstrated such potential. It is possible that four of the isolates (199, 481, 1138 and 1496) could be producers of an existing or completely new type of bacteriocin. Abundance (amount produced) and toxicity (killing capacity) are crucial parameters for practical application of bacteriocins. Extraction, purification and complete characterization of the compounds produced as well as comparison against Nisin should be carried out. Other bacterial species could also be challenged using the same methodology and culture collection described above.

Further studies

This work has shown the biotechnological potential of the Antarctic or sub-Antarctic Actinobacteria collection at University of Tasmania and future research is encouraged to make the best use of this resource. In the search for novel antibiotics, the traditional agar and zone of clearance assay is very labour intensive and it could be replaced by a faster and more reliable technology. In another collaboration with Mr. Jimmy Twin, the

use of chromatographic method to produce a faster high throughput detection method was considered as a possibility. The approach was to specifically identify the unusual amino acids Lanthionine and Methyllanthionine, often present in bacteriocins, in the supernatants of growth media after culture of Actinobacteria. If successful it would replace the traditional agar diffusion plate method with several advantages such as much faster results and lower minimum detection levels which in turn could potentially increase the number of positive hits.

Due to time constrains this project was not completed within the candidature, but the potential remains and should be pursued.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**:403-410.

Berdy, J. 2005. Bioactive microbial metabolites. *The Journal of Antibiotics* (Tokyo) **58**:1-16

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J. and Wheeler, D. L. 2003. *Nucleic Acids Research* **31**:23-27.

Chen, H. and Hoover D.G. 2003. Bacteriocins and their food applications. *Comprehensive Reviews in Food Science and Food Safety* **2**:82-100

DiMasi, J. A., Hansen, R. W. and Grabowski, H. G. 2003. The price of innovation: new estimates of drug development costs. *Journal of Health Economics* **22**:151–185.

Galtier, N., Gouy, M. and Gautier, C. 1996. *Computer Applications in the Biosciences* **12**:543-548.

Jack, R.W., Tagg, J.R and Ray, B. 1995. Bacteriocins of Gram-positive bacteria. *Microbiological Reviews* **59**:171-200

Jacobs, M. R. 2007. Retapamulin: a semisynthetic pleuromutilin compound for topical treatment of skin infections in adults and children. *Future Microbiology* **2**:591–600.

Klaenhammer, T.R. 1988. Bacteriocins of lactic acid bacteria. *Biochimie* **70**:337-349.

Lane, D. J. 1991. In *Nucleic acid techniques in bacterial systematics* (Eds, Stackebrandt, E. and Goodfellow, M.), John Wiley and Sons, New York, NY, pp. 115-175.

Leeb, M. 2004. Antibiotics: a shot in the arm. *Nature* **431**:892–893.

McAuliffe, O., Ross, R.P. and Hill, C. 2001. Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews* **25**:285-308

O'Brien, A., Sharp, R., Russell, N. J. and Roller, S. 2004. Antarctic bacteria inhibit growth of foodborne microorganisms at low temperatures. *FEMS Microbiology Ecology* **48**:157-167.

Raloff, J. 1998. Staging germ warfare in foods. *Science News* **153**:89-90.

Riley, M.A. and Wertz, J.E. 2002. Bacteriocins: Evolution, Ecology and Application. *Annual Review of Microbiology* **56**:117-137

Rodgers, S., Kailasapathy, K., Cox, J. and Peiris, P. 2002. Bacteriocin production by protective cultures. *Food Science Technology* **2**:59-68.

Twin, J. (2008). *Biodiscovery and biodiversity of Antarctic bacteria.* PhD Thesis, University of Tasmania. pp.137.

Chapter 4: Influence of high pressure processing on the composition of *Listeria monocytogenes* cell membrane lipids

INTRODUCTION

The idea of exposing food products to pressures above the atmospheric pressure for microbial preservation or inactivation purposes is not new. Hite (1899) studied the effect of pressure in the preservation of milk. He also pressure treated fruit and several other perishable products as well as tried to inactivate the tobacco mosaic virus using high pressure early in the 20th century (Giddings *et al* 1929). High pressure processing (HPP) or high hydrostatic pressure (HHP) consists of applying high pressures varying from 100 to 1000 MPa (987 to 9870 atm; 1 MPa = 9.87 atm) to liquid or solid products packed in flexible materials for seconds or minutes at time (Patterson 2005). This form of pasteurization is thought to deliver products with improved organoleptic properties and significantly less vitamin losses than conventional heat-based pasteurization treatments.

Critical process factors affecting HPP are treatment pressure, time at pressure, the time to reach maximum pressure and to return to ambient pressure, treatment temperature, initial product temperature, distribution of vessel temperature at pressure, product pH, a_w and product composition (Oxen and Knorr 1993; Heremans 1995; Heinz and Knorr 1999).

The item to be pressurized is submerged in liquid, usually water, and the pressure increased to achieve the pre-set processing value. Once the processing time is reached the vessel is depressurized back to the ambient pressure. The isostatic compression is uniform and equally distributed instantaneously throughout the chamber and products irrespective of size, shape or food composition (Smelt 1998). During the HPP processing it is estimated that sample temperature increases 3°C for each 100 MPa (USFDA 2000). This temperature increment is a consequence of the water adiabatic heating due to the pressure increase (first law of thermodynamics) and it will return to its original temperature after the decompression, i.e., adiabatic cooling (Hogan *et al* 2005).

Microorganisms show variable resistance to HPP, which is directly related to the amount of hydrostatic pressure applied. Stationary phase cells are more barotolerant than exponentially growing populations; spores are more tolerant than Gram-positive cells, which are more tolerant than yeasts. Gram-negative bacteria show the least resistance to the lethal effect of HPP (USFDA, 2000 and Shigehisa *et al,* 1991). The change in volume caused by the increased pressure affects the bacteria's biochemical and physiological primary processes as described by Cheftel (1995). Suzuki and Taniguchi (1972) suggested that below 100 MPa hydrophobic interactions between the proteins cause an increase in the reaction's volume whereas above 100 MPa the opposite occurs, that is a decrease in volume is experienced.

Microbial cell membrane, the primary site for pressure damage is mainly composed of a phospholipids bilayer integrated with proteins and is capable of regulating the transport of ions across it (San Martin *et al,* 2002). Membrane fatty acids can be divided into two major families, namely straight-chain fatty acids and branched-chain

fatty acids. The later comprises iso-, anteiso- and Ω-alicyclic fatty acids (Kaneda, 1991).

Pressurized membranes show altered permeability leading to the loss of intracellular constituents and an inability to control the movement of substances into and out of the cell resulting in inactivation. The conformation of proteins (including enzymes) may also be altered so that enzyme activity is enhanced, reduced or unaffected under pressure (Patterson, 2004).

Mastronicolis *et al* (1998) stated that *Listeria monocytogenes* cultured at 30°C is characterized by a high proportion of branched-chain fatty acids exceeding 85%, mainly anteiso-15:0 and anteiso-17:0. Kaneda (1991) emphasized that the fatty-acid profile of a bacteria is affected by temperature and other growth conditions, including branched-chain fatty acids. Studies of *L. monocytogenes* inactivation by HPP have indicated that there is considerable variation between strains, with some capable of surviving and displaying a "tailing" behavior after treatment with 800 MPa (Tay *et al* 2003). Mechanisms behind sub-lethal pressure inactivation are not well understood and need to be elucidated.

Several studies suggest that high pressure induces reversible or irreversible changes to the protein-lipid bilayer depending on the magnitude of the pressure applied. Kaneda (1991) also suggested the inclusion of branched-chain fatty acids into the cell membrane may prevent the liquid-to-gel transition and cause cell wall thickening. Hoover et al (1989) noted the membrane permeabilization caused by compression and reduction of the phospholipids bilayer while Kato and Hayashi (1999) suggested that high pressure causes the cell membrane to thicken under pressure making it less fluidic (more brittle) leading to breakage and protein denaturation and consequently

functionality losses. More recently Kato et al (2002) described high pressure induced changes at pressures lower than 100 MPa, between 100 and 220 MPa and above 220 MPa on biological membranes in general. This adaptive response of bacteria to high pressure is the objective of this study. I quantitatively and qualitatively evaluated the changes in the membrane fatty acids of *Listeria monocytogenes* when exposed to different magnitudes of high pressure and times as well as during the logarithmic and stationary growth phase.

MATERIAL AND METHODS

Culture conditions

Listeria monocytogenes FRRB 2542 was the strain used in this study. Originally isolated from salami it was obtained from CSIRO, Food Science Australia in Sydney. It was chosen among 9 other *Listeria monocytogenes* strains for its piezotolerance making it a perfect candidate for this experiment. In preparation for all experiments, one loopful of *Listeria monocytogenes* FRRB 2542 was transferred from glycerol stocks (stored at -80°C) to tryptone soya broth (TSB,10 ml, Oxoid) in duplicate and incubated at 37°C for 48 h, resulting in stationary phase cells (approximately 1 x 10^9 CFU/ml). One of the cell suspensions was diluted (0.1% peptone + 0.85% NaCl) to 1 x 10^5 CFU/ml and transferred (1 ml) to 12 separate flasks containing TSB (100 ml) resulting in an initial count of approximately 1 x 10^3 CFU/ml in each flask. The samples were incubated in a shaking water bath (Lauda, Germany) at 15°C with 35 rpm for 48 h (logarithmic phase) or 72 h (stationary phase). After 48 h of incubation liquid cultures from 6 flasks were aseptically transferred to the same sterile bag and manually homogenized by thorough mixing. Five samples (50 ml) were drawn and

transferred into sterile plastic pouches (Cryovac, Australia) and heat sealed excluding as much air as possible. Samples were then placed in a secondary bag filled with a sanitizing solution (peroxyacetic acid, 5000 ppm) and heat sealed to prevent any microbiological contamination inside the high pressure machine in case of any leakage. All samples were kept on ice before and after the pressure treatments for no more than 30 minutes.

Each pressure treated sample was decimally diluted (0.1% peptone + 0.85% NaCl), plated out in duplicate on tryptone soya agar with 0.6% yeast extract (TSA-Ye) and incubated at 37°C for 72h. The remaining cell suspension (approximately 48ml) for each treatment was filtered with a Whatman glass microfibre filter (no. 1825047, ϕ 47 mm, previously heated at 400°C for 24 h) and kept at -80°C for 2 weeks. The above experiment was repeated twice for flasks incubated for 48h and twice for flasks incubated for 72h, the latter yielding stationary growth phase cultures..

The samples were freeze-dried overnight on the filters and sent to University of Tasmania where I carried out for further FA analysis.

High Pressure Processing (HPP)

High pressure processing was carried out at Food Science Australia in Sydney, using a 2 L high pressure unit (Avure Technologies, USA). Water at ambient temperature of approximately 25°C was used as the pressure transmitting fluid. Samples were pressurized at either 450 or 600 MPa for 2 and 5 minutes. Control sample was kept on ice at atmospheric pressure (0.1 MPa).

The pressure come up time varied from 5 to 20 seconds depending on the pressure

magnitude whereas decompression time was almost instantaneous for all treatments. Table 1 below summarizes the processing parameters adopted.

Table 1 Combined pressure and time treatments applied to *Listeria monocytogenes* cells at both logarithmic and stationary phases.

Treatment No.	Pressure (MPa)	Time (min.)
1*	0.1	0
2	450	2
3	450	5
4	600	2
5	600	5

* Control (0.1 MPa = 1 atm)

Fatty Acid Analysis

Filters containing freeze dried pressure treated cells were extracted using a modified Bligh and Dyer solvent method (1959). A single phase extraction with methanol: chloroform: water (2:1:0.8 v/v/v) was used to yield a total solvent extract (TSE). An aliquot of TSE was trans-methylated by a solution of methanol: chloroform: hydrochloric acid (10:1:1, v/v/v) for 1 hour at 80°C. It was extracted three times using hexane: chloroform (4:1, v/v) to produce FAME (fatty acids methyl esters). Samples were supplemented with a known concentration of an internal standard (19:0 FAME) and analysed by gas chromatography (GC) using a Hewlett Packard 5890 II gas chromatograph and 5970A Mass Selective Detector

equipped with a 50 m x 0.22 mm internal diameter cross-linked methyl silicone (0.33 μm film thickness) fused-silica capillary column. Operating conditions were similar to those detailed in Nichols *et al.* (2000). Identification of fatty acid methyl esters from all samples was achieved by comparison of component spectra to those of known standards. Double bond position and geometry in monounsaturated isomers of selected samples were determined by the production and analysis of dimethyl disulphide adducts (Nichols *et al*, 1986).

RESULTS AND DISCUSSION

Microbial Inactivation

Listeria monocytogenes cells at both logarithmic and stationary phases were affected by all experimental conditions. As previously suggested (USFDA, 2000 and Shigehisa *et al,* 1991) cells at the stationary growth phase were more robust than cells at the logarithmic phase. For the stationary phase 1.1 and 2.1 log reductions were experienced for treatments of 450 MPa for 2 min and 450 MPa for 5 min, respectively compared to the control, whereas, the biggest logarithmic inactivation under the same treatments were 3.1 logs (control minus 450 MPa/5 min). In general, the higher the pressure and processing time the higher the inactivation achieved. In both growing phases and under the experimental conditions no growth was detected when pressure applied was 600 MPa independently of the exposure time to the HPP. Figure 1 illustrates those findings.

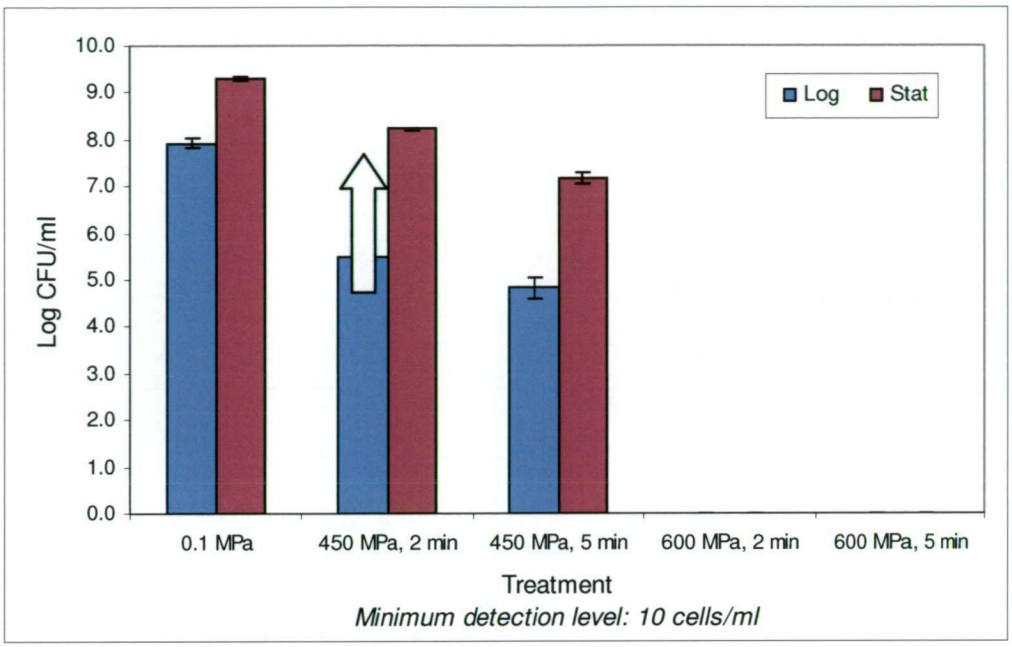


Figure 1 *Listeria monocytogenes* inactivation by HPP under different growth phase and processing conditions. The arrow above indicates the minimum CFU/ml for the cells grown at logarithmic phase and pressure treated at 450 MPa for 2 minutes. The highest dilution (-2) plated out for this treatment had counts above 300 (tntc). Minimum estimate was calculated by multiplying 300 (reliable count) x 100 (-2 dilution) x 10 (0.1 ml plated).

Log (300,000) = 5.48

Lipid Analysis

Pressure treated *Listeria monocytogenes* fatty acid profiles and relationships between branched and non-branched fatty acids for logarithmic and stationary growth phase are presented in Tables 2 to 5.

Table 2 Fatty acid composition of *L. monocytogenes* strain FRRB 2542 grown to exponential growth phase before and after high pressure processing treatment.

	Logarithmic Phase				
	Control	**450/2***	**450/5**	**600/2**	**600/5**
Fatty acid	%	%	%	%	%
n-12:0	1.1 (0.7)**	1.0 (0.2)	0.8 (0.1)	0.6 (0.1)	0.5 (0.1)
iso-14:0	1.8 (0.6)	0.0 (0.0)	0.0 (0.0)	0.8 (0.1)	0.8 (0.0)
n-14:0	2.2 (0.2)	2.0 (0.3)	1.2 (0.2)	1.7 (0.6)	1.7 (0.2)
iso-15:0	10.5 (0.3)	14.3 (0.8)	13.8 (0.5)	15.4 (0.3)	15.9 (0.7)
anteiso-15:0	61.4 (2.6)	68.4 (3.3)	69.1 (0.2)	62 (10.3)	60.8 (2.9)
iso-16:0	3.8 (0.4)	1.2 (0.3)	1.5 (0.1)	1.4 (0.5)	2.1 (0.2)
n-16:0	6.6 (2.2)	3.7 (1.8)	3.6 (0.4)	6.5 (4.1)	6.0 (2.2)
iso-17:0	2.2 (1.2)	1.2 (0.4)	0.9 (0.1)	1.2 (0.1)	1.4 (0.2)
anteiso-17:0	6.8 (1.0)	6.9 (0.4)	7.4 (0.3)	7.3 (1.7)	8.6 (0.6)
n-18:0	3.7 (1.2)	1.3 (0.9)	1.7 (0.9)	3.2 (3.0)	2.2 (0.7)
branched/non-branched	6.4	11.6	12.8	7.3	8.6
average carbon chain length	15.3	15.2	15.2	15.3	15.3
non-branched (%)	13.6	8.0	7.2	12.0	10.4
iso (%)	18.3	16.7	16.3	18.7	20.3
anteiso (%)	68.1	75.3	76.5	69.3	69.3
Total branched (%)	86.4	92.0	92.8	88.0	89.6

*Pressure (MPa)/minutes of exposure.

** Numbers inside parentheses are standard deviations

Table 3 Fatty acid composition of *L. monocytogenes* strain FRRB 2542 grown to stationary growth phase before and after high pressure processing treatment.

	Stationary Phase				
	Control	**450/2**	**450/5**	**600/2**	**600/5**
Fatty acid	%	%	%	%	%
n-12:0	0.6 (0.3)**	0.4 (0.0)	0.4 (0.1)	0.4 (0.1)	0.4 (0.0)
iso-14:0	1.0 (0.2)	0.8 (0.0)	0.8 (0.1)	0.9 (0.1)	0.8 (0.0)
n-14:0	2.4 (0.4)	2.1 (0.3)	2.2 (0.2)	2.4 (0.0)	2.8 (0.3)
iso-15:0	18 (0.3)	17.6 (0.2)	17.3 (0.5)	17 (0.3)	16.3 (0.5)
anteiso-15:0	66.8 (0.5)	68.3 (1.5)	67.3 (2.2)	65.9 (1.8)	63.8 (3.9)
iso-16:0	1.3 (0.3)	1.2 (0.2)	1.2 (0.2)	1.3 (0.1)	1.1 (0.0)
n-16:0	2.1 (0.8)	1.7 (0.9)	2.3 (0.3)	3.4 (1.0)	5.9 (2.6)
iso-17:0	1.1 (0.1)	0.9 (0.1)	0.9 (0.2)	0.9 (0.3)	0.9 (0.0)
anteiso-17:0	6.1 (0.3)	6.6 (0.3)	6.5 (0.2)	6.3 (0.1)	6.1 (0.2)
n-18:0	0.6 (0.8)	0.4 (0.6)	1.2 (0.4)	1.6 (0.8)	2 (1.6)
branched/non-branched	16.6	21.0	15.6	12.1	8.0
average carbon chain length	15.1	15.1	15.2	15.2	15.2
iso (%)	21.4	20.6	20.2	20.1	19.0
anteiso (%)	72.9	74.9	73.8	72.2	69.9
Total branched (%)	94.3	95.5	94.0	92.3	88.9

*Pressure (MPa)/minutes of exposure.

** Numbers inside parentheses are standard deviations

The role of fatty acids in the membrane adaptation of *Listeria monocytogenes* to stresses such as low and high temperature and acidic pH is well documented and discussed in the next paragraphs. There is still a need for better understanding of responses to high pressure at the membrane level and the associated fatty acid adaptations. Similarities or differences in the fatty acid profiles of pressure treated cells in comparison with other stresses will give us an insight into similarity or differences in these responses. Under normal growing conditions at 37°C *Listeria monocytogenes'* fatty acid membrane is mainly composed of branched-chain fatty acids iso-15:0, anteiso-15:0 and anteiso-17:0 that combined represent more than 85% of this bacterium's fatty acids (Annous *et al* 1997; Mastronicolis *et al* 1998). Anteiso-15:0 alone is responsible for up to 70% of the total branched-chain fatty acids. Lipid compositions from the barotolerant strain are in agreement with those findings. When growth temperature is below 7°C *Listeria monocytogenes* switches from iso-15:0 to anteiso-15:0 and decreases its amount of anteiso-17:0. This cold adaptation has been described by Annous *et al* (1997) and Puttmann *et al* (1993), amongst others.

Listeria monocytogenes membrane fatty acid adaptation to pH stress was studied by Giotis *et al* (2007) and Moorman *et al* (2008). In their study reduced levels of the branched-chain fatty acid anteiso- form was found to occur causing a decrease in membrane fluidity and an increase in cell hydrophobicity. They also suggested that the balance between anteiso- and iso- fatty acids was more important to pH adaptation than the different levels of saturation and abundance of the fatty acids. Moorman *et al* (2008) did not observe significant changes to membrane fluidity or cell hydrophobicity in heat stressed *Listeria innocua* cells that were cultured on TSB-Ye

for 8h at 37°C and then briefly exposed to 45°C for an additional 1h.

Being by far the most abundant branched-chain fatty acids in *Listeria monocytogenes* it was expected the major three fatty acids iso-15:0, anteiso-15:0 and anteiso-17:0 would play an important role in adaptation mechanisms to high pressure processing as it did for the others stresses discussed above.

Under the experimental conditions *Listeria monocytognenes* FRRB 2542 reacted to the increased pressure by altering its iso branched-chain fatty acids only. Significant changes in average acyl chain length and anteiso/iso branching ratios did not occur possibly due to the low temperature (15°C) the cells were initially grown at. Statistically significant fatty acid changes during logarithmic phase were found for iso-14:0 ($p<0.0045$), iso-15:0 ($p<0.0013$), iso-16:0 ($p<0.0028$) and for total iso-branched fatty acids ($p<0.0093$), in response to the pressurized conditions. Iso-15:0 was the only fatty acid presenting significant differences during the stationary growth phase ($p<0.0376$). These findings, compared with the ones described above, differ from adaptation responses of *Listeria monocytogenes* membrane fatty acids to cold, heat and pH stress though it is unknown what effect high pressure would have on cells grown at an incubation temperature higher than what was done in these experiments.

The mechanisms invoked by *L. monocytogenes* to deal with increased pressures could be a consequence of the inactivation of enzymes involved in lipid biosynthesis as suggested by Jaenicke *et al* (1990). In other words, the changes in the membrane fatty acids (or lack of changes) is not necessarily a bacterial adaptation to HPP but a consequence of the inactivation of the enzymes involved in lipid biosynthesis

(inactivated enzyme = no activity = no lipid production or membrane maintenance). The resultant increase in gene expression for fatty acid biosynthesis could be a compensation for this inactivation or inhibition. Future work must be carried out to further elucidate the effect of high pressure on bacterial cell membrane composition coupled with enzymatic inactivation under a broader range of conditions.

Through recent published work between myself and my supervisors (Bowman *et al* 2008; *see also* Chapter 5) gene expression data determined using DNA microarray analysis is available for strain FRRB 2542 exposed to 400 and 600 MPa for 5 minutes (compared to atmospheric pressure controls). That study aimed to identify the genes upregulated or downregulated during the high pressure processing hoping to elucidate some of the adaptive pathways used by this bacterium to cope with the increased pressure. Utilising this data several genes were found that are responsive to high pressure treatments. These included genes coding proteins for the fatty acids biosynthesis pathway as well as branched-chain amino acid biosynthesis and metabolism as shown in Table 4 below.

The gene expression data suggests that fatty acid biosynthesis increases as almost all genes for the pathway are upregulated. Presumably HPP induces the cells to make more fatty acids to repair membranes or to replace membrane sections. A similar response was found for *E. coli* in the late log phase but not early log phase when exposed to 50 MPa (Ishii et al. 2005). The gene that suggests this trend the most is acpP (expression increased >5-10-times), which codes the acyl carrier protein, critical for lipid biosynthesis and carries the finished product to other enzymes that build the lipid bilayer (phospholipid acyltransferases etc.). There is also evidence that branched

chain amino acid (BCAA) biosynthesis increases. BCAAs are needed for the synthesis of branched chain fatty acids (BCFA). The gene that codes the important step in this process, branched chain amino acid aminotransferase (BCAT, lmo0978), has slightly increased expression after pressure treatments (Table 4).

Table 4 Up and downregulated *L. monocytogenes* FRRB 2542 genes under high pressure

FATTY ACID BIOSYNTHESIS PATHWAY

Gene no.	400 Mpa/ 5 min	600 Mpa/ 5 min	Gene	Function	E.C. code	Pathway
	Expression change (log_2):					
lmo1810	2.17*	2.64	*fapR*	putative fatty acid biosynthesis regulator		Transcriptional regulation, Fatty acid biosynthesis
lmo1356	1.64	0.7	*accB*	acetyl-CoA carboxylase biotin carboxyl carrier protein		Fatty acid biosynthesis, Pyruvate metabolism, Propanoate metabolism;Biotin metabolism
lmo1573	1.92	1.41	*accD*	acetyl-CoA carboxylase carboxyl transferase subunit beta	6.4.1.2B	Fatty acid biosynthesis, Pyruvate metabolism, Propanoate metabolism
lmo1806	3.32	2.38	*acpP*	acyl carrier protein		Propanoate metabolism
lmo1808	1.07	0.71	*fabD*	malonyl CoA-acyl carrier protein transacylase	2.3.1.39	Fatty acid biosynthesis
lmo0354	0.73	0.45	*fadD*	putative acyl-CoA synthetase (long-chain-fatty-acid--CoA ligase)	2.3.1.39	Fatty acid biosynthesis
lmo2201	0.7	1.31	*fabF*	3-oxoacyl-[acyl-carrier-protein] synthase I/II	2.3.1.179	Fatty acid biosynthesis
lmo2202	3.49	1.93	*fabH*	3-oxoacyl-[acyl-carrier-protein] synthase III	2.3.1.180	Fatty acid biosynthesis, Branched chain fatty acid biosynthesis

lmo1807	0.74	0.38	*fabG*	3-oxoacyl-[acyl-carrier protein] reductase	1.1.1.100	Fatty acid biosynthesis
lmo0970	1.6	0.6	*fabI*	enoyl-[acyl-carrier protein] reductase (NADH)	1.3.1.9	Fatty acid biosynthesis
lmo1688	-1.39*	-0.31	*fabL*	enoyl-[acyl carrier protein] reductase III	1.3.1	Fatty acid biosynthesis

OTHER FATTY ACID METABOLISM-RELATED

lmo0489	-1.86	-1.5	*fadH*	putative 2,4-dieonyl-CoA reductase, FMN-linked	1.3.1.34	Fatty acid biosynthesis, Fatty acid metabolism
lmo1414	2.65	2	*atoB*	acetyl-CoA acetyltransferase	2.3.1.9	Pyruvate metabolism, Propanoate metabolism, Butanoate metabolism, Branched-chain amino acids metabolism, Fatty acid metabolism, Other amino acid metabolism
lmo2235	1.58	1.32	*enr*	putative 2-enoate reductase, NADH-dependent flavin oxidoreductase; similar to Enr of Clostridium spp. (e.g. butenoate converted to butanoate)	1.3.1.31	Butanoate metabolism, Fatty acid metabolism

BRANCHED-CHAIN AMINO ACID BIOSYNTHESIS

lmo1983	0.35	0.79	*ilvD*	dihydroxy-acid dehydratase	4.2.1.9	Branched-chain amino acids biosynthesis, Pantothenate biosynthesis, Coenzyme A (CoA) biosynthesis; Isoleucine biosynthesis, Valine biosynthesis
lmo1985	1.06	0.73	*ilvH*	acetolactate synthase small subunit	2.2.1.6	Branched chain amino acids biosynthesis; Isoleucine biosynthesis, Valine biosynthesis, Pantothenate biosynthesis, Coenzyme A (CoA) biosynthesis

lmo1986	2	0.9	*ilvC*	ketol-acid reductoisomerase	1.1.1.86	Branched-chain amino acids biosynthesis, Pantothenate biosynthesis, Coenzyme A (CoA) biosynthesis; Isoleucine biosynthesis, Valine biosynthesis
lmo1988	1.98	0.17	*leuB*	3-isopropylmalate dehydrogenase	1.1.1.85	Branched-chain amino acids biosynthesis
lmo1989	1.83	1.22	*leuC*	3-isopropylmalate dehydratase large subunit	4.2.1.33B	Pyruvate metabolism, Branched chain amino acids biosynthesis, Leucine biosynthesis
lmo1990	1.14	0.82	*leuD*	3-isopropylmalate dehydratase small subunit	4.2.1.33B	Pyruvate metabolism, Branched chain amino acids biosynthesis, Leucine biosynthesis
lmo1991	0.79	0.87	*ilvA*	threonine dehydratase	4.3.1.19	Branched-chain amino acids biosynthesis, Glycine/serine/threonine metabolism, Isoleucine biosynthesis
lmo1992	-1.55	-1.58	*alsD*	alpha-acetolactate decarboxylase	4.1.1.5	Butanoate metabolism, C5-Branched dibasic acid metabolism
lmo2006	-2.71	-1.46	*alsS*	alpha-acetolactate synthase	2.2.1.6	Butanoate metabolism, C5-Branched dibasic acid metabolism

BRANCHED-CHAIN AMINO ACID METABOLISM (for branched-chain fatty acids)

lmo0978	0.58	0.61		branched-chain amino acid aminotransferase (BCAT)	2.6.1.42	Branched-chain amino acids metabolism; Branched-chain amino acids biosynthesis; Pantothenate biosynthesis; Coenzyme A (CoA) biosynthesis; Branched-chain fatty acid biosynthesis; Isoleucine biosynthesis; Leucine biosynthesis; Valine biosynthesis

lmo1369	1.05	0.35	*ptb*	phosphate butyryltransferase	2.3.1.19	Butanoate metabolism
lmo1370	0.47	0.79	*buk*	butyrate kinase	2.7.2.7	Butanoate metabolism
lmo1374	-1.33	-0.66	*bkdB*	branched-chain alpha-keto acid dehydrogenase E2 subunit (lipoamide acyltransferase)	2.3.1.168	Branched-chain amino acids metabolism, Butanoate metabolism, Branched-chain fatty acid biosynthesis

*** Red = significant increase p<0.01, Green = significant decrease p<0.01**

In other unpublished data (John Bowman pers. comm.) genes in the fatty acid biosynthesis pathway exhibit increased expression at low temperature (4°C vs 25°C) but not at pH 5. BCAA synthesis gene expression increases were not observed at low temperature possibly owing to the reduced metabolic rate. So in some ways the responses to HPP overlap what happens at low temperature but since cells of FRRB 2542 were cultivated at 15°C the responses to fatty acid changes are probably relatively muted compared to what has been found in other studies in which different growth conditions are compared (e.g. 10°C vs 37°C, Zhu et al. 2005). This is suggested by a lack of fatty acid acyl chain length shortening (Tables 2 and 3) a major response to fatty acid composition by HPP in various bacteria (Martinez-Rodriguez & Mackey 2005). Also the high pressure exposure times were very short and at 600 MPa the cells are injured very rapidly (as suggested by inactivation after just 2 min, Fig. 1) thus the cell population responses would be likely to be different to more survival/growth permissive conditions.

AKNOWLEDGEMENTS

Claudio Bittencourt was supported by a top up CSIRO, Food Science Australia scholarship. Authors thank B. Chapman, M. Bull and C. Stewart for their help with the HPP experiments in North Ryde, Sydney.

At the University of Tasmania I wish to thank T. Lewis, D. Nichols, M. Miller and D. Ratkowsky for their contribution. Special thanks to Assoc. Prof. John Bowman for his always valuable comments and promptness every time it was requested.

REFERENCES

Annous, B.A., Becker, L.A., Bayles, D.O., Labeda, D.P. and Wilkinson, B.J. 1997. Critical role of anteiso-C-15:0 fatty acid in the growth of *Listeria monocytogenes* at low temperatures. *Applied and Environmental Microbiology* **63**:3887-3894.

Bligh, E.G., and Dyer, W.J. 1959. A rapid method for total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37:**911-917.

Bowman, J.P., Bittencourt, C. and Ross, T. 2008. Differential gene expression of *Listeria monocytogenes* during high hydrostatic pressure processing. *Microbiology* **154**: 462-475.

Cheftel, J.C. 1995. High pressure, microbial inactivation and food preservation. *Food Science and Technology International* **1**:75-90

Giddings, N.T, Allard, H.A. and Hite, B.H. 1929. Inactivation of the tobacco mosaic virus by high pressure. *Phytopathology* **19**:749-750.

Giotis, E.S., McDowell, D.A, Blaie, I.S. and Wilkinson, B.J. 2007. Role of branched-chain fatty acids in pH stress tolerance in *Listeria monocytogenes. Applied and Environmental Microbiology* **73**:997-1001.

Heinz, V. and Knorr, D. 1999. The effects of high pressure on microbial spores. *European Conference on Emerging Food Science and Technology.* Tempere, Finland. November 22-24 1999.

Heremans, K. 1995. High pressure effects on biomolecules. *High Pressure Processing of Foods. Leicestershire*, UK. Nottingham University Press.

Hite, B.H. 1899. The effect of pressure in the preservation of milk. *Bulletin of West*

Virginia University of Agricultural Experiment Station **58**:15-35.

Hogan, E., Kelly, A.L. and Sun, D-W. 2005. High pressure processing of foods: an overview. *Emerging Technologies for Food Processing* (Sun Da Wen, ed) pp. 3-31. Academic Press.

Hoover, D.G., Metrick, C., Papineau, A.M., Farkas, D.F. and Knorr, D. 1989. Biological effects of high hydrostatic pressure on food microorganisms. *Food Technology* **43**:99-107

Ishii, A., Oshima, T., Sato, T., Nakasone, K., Mori, H. & Kato, C. 2005. Analysis of hydrostatic pressure effects on transcription in *Escherichia coli* by DNA microarray procedure. *Extremophiles* **9**:65-73.

Jaenicke, R and Zavodszky, P. 1990. Proteins under extreme physical conditions. *FEBS Letters* **268**:344-349

Kaneda, T. 1991. Iso- and anteiso-fatty acids in bacteria: biosynthesis, function and taxonomic significance. *Microbiological Reviews* **55**:288-302.

Kato, M. and Hayashi, R. 1999. Effects of high pressure on lipids and biomembranes for understanding high pressure induced biological phenomena. *Bioscience, Biotechnology and Biochemistry* **63**:1321-1328

Kato, M., Hayashi, R., Tsuda, T. and Taniguchi, K. 2002. High pressure-induced changes of biological membrane. *European Journal of Biochemistry* **269**:110-118

Martinez-Rodriguez, A. and Mackey, B.M. 2005. Physiological changes in Campylobacter jejuni on entry into stationary phase. *International Journal of Food Microbiology* **101**:1-8

Mastronicolis, S.K., German, J.B., Megoulas, N., Petrou, E., Foka, P. and Smith, G.M.

1998. Influence of cold shock on the fatty-acid composition of different lipid classes of the food-borne pathogen *Listeria monocytogenes*. *Food Microbiology* **15**:299-306.

Moorman, M., Thelemann, C.A., Zhou, S., Pestka, J., Linz, J.E. and Ryser, E.T. 2008. Altered hydrophobicity and membrane composition in stress-adapted *Listeria innocua*. *Journal of Food Protection* **71**:182-185.

Nichols, P.D., Guckert, J.B. and White, D.C. 1986. Determination of monounsaturated fatty acid double-bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts. *Journal of Microbiological Methods* **5**:49-55.

Nichols, D.S., Olley, J., Garda, H., Brenner, R.R and McMeekin, T.A. 2000. Effect of temperature and salinity stress on growth and lipid composition of *Shewanella gelidimarina*. *Applied and Environmental Microbiology* **66**:2422-2429.

Patterson, M. 2004. Under pressure: a novel technology to kill micro-organisms in foods. *Culture* **24**:2.

Patterson, M.F. 2005. Microbiology of pressure-treated foods. *Journal of Applied Microbiology* **98**:1400-1409.

Puttmann, M., Ade, N. and Hof, H. 1993. Dependence of fatty acid composition of *Listeria* spp. on growth temperature. *Research in Microbiology* **144**:279-283.

Oxen, P. and Knorr, D. 1993. Baroprotective effects of high solute concentrations against inactivation of *Rhodotorula rubra*. *Lebensmittel-Wissenschaft und Technologie* **26**:220-223.

San Martin, M.F., Barbosa-Canovas, G.V. and Swanson, B.G. 2002. Food processing by high hydrostatic pressure. *Critical Reviews in Food Science and Nutrition* **42**:627-645.

Shigehisa, T., Ohmori, T., Saito, A., Taji, S. and Hayashi, R. 1991. Effects of high hydrostatic pressure on characteristics of pork slurries and inactivation of microorganisms associated with meat and meat products. *International Journal of Food Microbiology* **12**:207-216.

Smelt, J.P.P.M. 1998. Recent advances in the microbiology of high pressure processing. *Trends Food Science and Technology* **9**:152-158.

Suzuki, K. and Taniguchi, T. 1972. Effect of pressure on biopolymers and model systems. In: M. A. Sleigh and A. G. Macdonald (eds). *The Effect of Pressure on Living Organisms*. New York. Academic Press Inc. 103.

Tay, A., Shellhammer T.H., Yousef A.E. and Chism, G.W. 2003. Pressure death and tailing behavior of *Listeria monocytogenes* strains having different barotolerances. *Journal of Food Protection* **66**:2057-2061.

USFDA, 2000. Kinetics of microbial inactivation for alternative food processing technologies. High Pressure Processing. Center for Food Safety and Applied Nutrition.

Zhu, K., Ding, X., Julotok, M. and Wilkinson, B.J. 2005. Exogenous isoleucine and fatty acid shortening ensure the high content of anteiso-C15:0 fatty acid required for low-temperature growth of *Listeria monocytogenes. Applied and Environmental Microbiology* **71**:8002-8007

Chapter 5: Differential Gene Expression of *Listeria monocytogenes* During High Hydrostatic Pressure Processing

This thesis chapter represents a collaborative work between primary supervisor (Assoc. Prof. Tom Ross), Assoc. Prof. John Bowman, and myself.

For this series of experiments I made important contributions.

I carried out the experimental design and execution of it, including cultivation and high pressure processing, enumeration, death curves, mRNA extraction and purification. Assoc. Prof. John Bowman carried out the data analysis and preparation of the manuscript.

This project was fairly complex and required in-depth molecular microbiology expertise. Assoc. Prof. John Bowman was a perfect fit for it with his extensive molecular knowledge.

I particularly enjoyed working and learning from him.

The chapter constitutes a manuscript that was published in late 2008 in the journal Microbiology (SGM) Volume **154**, pages 462-475.

Chapter 6: Final Discussion and Conclusion

Listeria monocytogenes is a facultative anaerobic pathogen found in soil and water, on vegetation, food contact surfaces and in raw food materials including seafood. It grows at refrigeration temperatures and up to 14% sodium chloride making it almost impossible to control in fresh or minimally-processed seafood under aerobic or anaerobic storage conditions. This thesis has considered strategies to control growth of this bacterium on seafood using both established and novel non-thermal technologies and sought to elucidate physiological mechanisms underlying one of the approaches, namely high pressure processing.

General seafood spoilage and safety as well as non-thermal technologies currently used or with potential to be utilized by the seafood industry was the focus of the first chapter which summarises existing relevant knowledge to be read in conjunction with the subsequent experimental results and discussions. In this thesis I have attempted to integrate knowledge of seafood microbiology, including spoilage microbiota and pathogens, with a practical industry perspective to link applications with the relevant science. In many ways this reflects the several years that I spent working as a technical and production manager within the Australian seafood processing industry.

Chapter 2 describes an evaluation of the efficacy of three commercially available antimicrobial products to inhibit the growth of *Listeria monocytogenes* on chilled vacuum packed cold smoked salmon (CVPCSS) stored at 4 and 10°C for a period of up to 40 days. The products evaluated were *PURASAL Opti.Form4* at 1.5 and 3% w/w (Purac, liquid solution, 56% potassium lactate and 4% sodium diacetate, ratio 14:1

w/w), *PURASAL Powder S 96* at 2.3% w/w (Purac, powder, 96% sodium lactate) and *GUARDIAN NR100* at 0.1% w/w (Danisco, powder, 1.25% Nisin and min. 65% natural rosemary extract). I also organised and oversaw organoleptic evaluations of the untreated and treated samples in parallel with microbial enumerations to investigate the potential of those three products as shelf-life extenders.

Both *PURASAL* products were successful in inhibiting *L. monocytogenes* growth. *PURASAL S96* (2.34% w/w) at 4°C completely stopped *L. monocytogenes* growth: the initial two log cfu/g remained unchanged until the end of the experiment on day 30. *L. monocytogenes* on control samples for the same conditions had increased by 3.7 logs at day 30. Samples kept at 10°C for 35 days also showed that the S96 antimicrobial product reduced the ability of *L. monocytogenes* cells to grow. Treated and untreated samples experienced 1.9 and 5.3 logs of growth respectively by day 35.

PURASAL OptiForm4 (1.5% w/w) also inhibited completely the growth of *L. monocytogenes* on treated samples kept at 4°C for 40 days, whereas untreated samples increased five logs during the same period of time. A 1.2 and 5.1 log increase was recorded for treated and untreated samples respectively when stored at 10°C. When the concentration of *OptiForm4* was increased to 3% and treated samples kept at 10°C it produced a listericidal effect and counts were reduced from 3.3 logs on day zero to 1.3 logs at day 45.

GUARDIAN NR100 appears to have failed to penetrate (i.e. dissolve into) the flesh of the salmon fillets: no growth inhibition was noted on either treated nor untreated sliced cold smoked salmon samples kept at 4 and 10°C for 35 days.

Under the experimental conditions employed none of the three compounds, regardless of concentration used and storage temperature, were able to inhibit the growth of the

microflora naturally present on cold smoked salmon. A simplistic approach to correlate the total number of bacteria with the product shelf-life was chosen for this experiment. It is acknowledged that other chemical and physical measures such as those described in Chapter 1 should be undertaken in order to describe the spoilage scenario fully. It was used as a first estimate only, based on the pragmatic consideration of legislated prescriptions of quality based on TPC, but revealed no significant differences between antimicrobial treatments and relevant untreated controls.

No statistically significant difference was found by the sensorial panel with regards to the sensorial attributes (color, taste and overall acceptance) of cold smoked salmon treated with *PURASAL S96* when compared with untreated cold smoked salmon.

Because of its powder form, PURASAL S96 possesses an advantage over the liquid *PURASAL OptiForm4* because it can be directly added to the curing salts and applied over the raw salmon fillets. With the liquid solution it has to be injected as a mixture with the brine solution and this would increase the risk of contamination or even potentially "inoculate" the deeper tissue with microbial contaminants from the fish surface or from the brine solution itself, especially if it is recycled.

The above information presents PURASAL S96 as an ideal listeriostatic candidate to be added to vacuum packed chilled cold smoked salmon.

The research continued in a "biodiscovery" oriented direction, essentially looking for novel antimicrobial agents to inhibit *L. monocytogenes* that could potentially be incorporated into or applied to the surface of food products. This exploration involved screening of Antarctic and sub-Antarctic soil-associated bacteria for potentially novel listericidal bacteriocins (described in Chapter 3). Approximately 1600 actinobacteria

isolates belonging to the University of Tasmania cryogenic archive were screened against five different *Listeria monocytogenes* strains including the reference strain Scott A for their ability to produce antimicrobial compounds. The great majority (75%) of the collection were previously morphologically identified as *Streptomyces*, the largest antibiotic-producing genus in the microbial world (Berdy, 2005).

Traditional agar diffusion and zone of clearance assays were selected for this large experiment. The first preliminary screen yielded 200 isolates that were capable of inhibiting one or more *L. monocytogenes* strains. From those 200, 20 were able to inhibit all five *L. monocytogenes* strains included in the screening protocol. They became the focus of the subsequent research. A partial characterization of the compounds was undertaken using proteolytic enzymes. This led to the assumption that the antimicrobial compounds were bacteriocins, which are peptides. Compounds produced by strains 199, 481, 1138 and 1496 were degraded by the enzymes as suggested by reduced activity following protease exposure. Twelve of the 20 antimicrobial producers identified by 16S rRNA sequencing presented unique sequences. A phylogenetic tree was developed to show their homology along with other strains from the genus *Streptomyces*.

The investigation demonstrated that Antarctic or Sub-Antarctic soils harbour potentially useful antimicrobial producers with the capacity to target pathogenic species and have potential applications for food safety. It is possible that the four isolates (199, 481, 1138 and 1496) identified as most promising could produce novel bacteriocins and be the subject of more detailed study in the future.

Abundance (amount produced), toxicity (killing capacity), specificity and "cold activity", i.e. being able to challenge or inactivate cold loving bacteria on refrigerated

food products such as ready-to-eat seafood, are crucial parameters when it comes to bacteriocins for application to these types of foods. Extraction, purification and complete characterization of the compounds produced as well as comparison against the benchmark bacteriocins, i.e. Nisin, should be carried out as well as assessment of potential toxicity to human consumers. Other genera or species could also be challenged using the methodology, including strains from the culture collection described above. The results presented support bio-prospecting strategies for antimicrobials against pathogen of human concern that include species from extreme environments.

The development of a faster, more reliable and less subjective high throughput screening method could greatly increase the potential to discover new active biological compounds with antimicrobial properties. It is envisaged that a chromatographic method specifically looking for biological markers, such as the unusual amino acids lanthionine and methyllanthionine often present in lantibiotics, would make the screening task much faster and accurate and in turn more samples could be processed.

Following on from the antimicrobial oriented work there was great interest in investigating the utility of high pressure processing (HPP) as a means to inhibit or destroy *L. monocytogenes*. Though it was quickly realised that HPP is highly inappropriate for processing of salmon or other finfish products there was an interest in discovering responses *L. monocytogenes* have to HPP from a more physiological point of view since little data was available in the literature. Reports have shown *L. monocytogenes* to be potentially quite resistant to HPP and even recover from HPP-induced damage. Chapter 4 explores whether the cell membrane is an important mediator of the effects of HPP and reports the changes occurring in the fatty acid

composition of the membrane in response to HPP (450 MPa and 600 MPa). The results suggested that under pressure, irrespective of the growth phase, *Listeria monocytogenes* responds by changing the abundance of iso branched-chain fatty acid of its cell membrane. This fatty acid adaptive response is different to that caused by cold, pH or heat stresses. *L. monocytogenes* cells for this experiment were grown at 15°C but the effects of HPP on *L. monocytogenes* membrane fatty acids grown at higher temperatures remains unknown. An experiment covering a wide range of growth temperature as well as different HPP processing times and pressures would help to elucidate this question. Additionally, the work decribed in Chapter 4 notes that several genes, related to the synthesis of fatty acids and more specifically branched-chain fatty acids, that were upregulated or downregulated during the HPP, providing a starting point to better elucidate short term responses to mechanical stress to cells. The microarray data (presented in Chapter 5) suggests that fatty acid bisoynthesis increases and is supported by the upregulation of the majority of the genes involved in the biosynthetic pathway. There were also increases in the genes involved in branched-chain amino acids (leucine, valine and isoleucine) biosynthesis, which act as primers for the synthesis of branched-chain fatty acids. Following on from this specific focus in Chapter 5 the observations made were broadened with DNA microarray technology used to assess changes in overall gene expression of *L. monocytogenes* when exposed to HPP. Strain 2542, grown at 15°C to the exponential phase was subjected to 400 and 600 MPa for 5 min. Expression changes were analysed using gene set enrichment and the data was used to predict the responses of aspects of the cell to HPP induced cell injury. HPP induced increased expression of genes associated with DNA repair mechanisms, transcription and translation protein complexes, the septal ring, the

general protein translocase system, flagella assemblage and chemotaxis, and lipid and peptidoglycan biosynthetic pathways. On the other hand, HPP repressed genes associated with catabolism and virulence. It was also observed HPP strongly represses the SigB and PrfA regulons. The overall conclusions were that HPP seems to invoke a cell maintenance state in which compensation against HPP induced dissociation protein complex occurs but strongly suppresses catabolism and virulence activity that are more associated with active growth processes. Further work is needed to determine in more detail whether HPP induced cell injury can lead to a "repair mode" and whether cells that have been inactivated by HPP can actually eventually recover to regrow. This would involve investigating different strains including mutants under a range of mild to harsh HPP exposures with more of a focus on defining inactivation and subsequent recovery processes.

Future Research

To be able to use lower magnitudes of high pressure processing some researchers are taking advantage of the "multi-hurdle" approach and the synergistic effects that often come hand in hand when two or more technologies are mixed together. For example, Lee *et al* (2003) demonstrated that the addition of Nisin to liquid egg samples before high pressure processing increased the listericidal effect of HPP on *Listeria seeligeri* by five logs. Interestingly, there were no significant differences in *Listeria* reduction when both interventions were applied individually, clearly indicating the synergistic effect between them.

A similar effect to the one described above was achieved when HPP was applied in conjunction with a new bacteriocin produced by *Lactobacillus casei* in order to

inactivate *Listeria monocytogenes* (Chung *et al*, 2003). The combination of bacteriocin and HPP (350 MPa x 1 minute) delivered a four log reduction, whereas individually each treatment was only capable of achieving one log reduction.

Another possible advantage of combining HPP and bacteriocins is the possibility of using HPP as the first hurdle stage to make the bacterial spore germinate providing access for the bacteriocin (second stage hurdle) to "work its magic" against the vegetative and less robust microbial cell. The principle in some ways would be similar to Tyndallization (use of heat to induce spore germination followed by an additionally heat treatment 48 h later to kill the vegetative cell) but without the use of heat, a "Cold Tyndallization". If the first stage of the above proposed approach can be less aggressive (lower high pressure levels, ie. 100-150 MPa) then it would certainly become a very attractive and robust way to damage or inactivate bacterial spores without imparting irreversible and unwanted sensorial changes to raw like or minimally processed seafood products. Further studies are required to investigate the multi-hurdle approach suggested above as well as its applications and limitations to the seafood industry.

REFERENCES

Berdy, J. 2005. Bioactive microbial metabolites. *J. Antibiot.* (Tokyo), **58**:1-16

Chung, H.J., Tay, A., Yousef, A.E., Shellhammer, T.H. and Chism. G.H. 2003. Combination effect of bactericoin and high pressure processing on inactivation of *Listeria monocytogenes* strains. Food Microbiology: Control of foodborne microorganisms by antimicrobials, IFT Annual Meeting, Session 29F, Chicago

Lee, D.U., Heinz, V. and Knorr, D. 2003. Effects of combination treatments of Nisin and high-intensity ultrasound with high pressure on the microbial inactivation in liquid whole egg. *Innovative Food Science and Emerging Technologies,* **4**:387-393

Appendix 1: Sensorial Analysis Statistics

Sodium Lactate

SAS System

Class Level Information

Class	Levels	Values
Tasters	5	1, 2, 3, 4 and 5
Treatments	2	Control and Na-L

Day	0	3	7	10	21	30
Colour	0.4525	0.0176	0.3239	0	0.3239	0
Taste	0.3942	0.1655	0.0461	0.2944	0.1799	0.5211
Overall	0.3942	0.1019	0.0677	0.1943	0.0207	0.8931

Score	Description
1	Hated
2	Extremely Disliked
3	Disliked
4	Neutral
5	Liked
6	Extremely Liked
7	Loved

Appendix 2: Actinobacteria x *Listeria monocytogenes* screen

L1	FRRW 2343
L2	FRRW 2345
L3	FRRB 2472
L4	FRRB 2542
L5	L5-22

Actino no.	**L1**		**L2**		**L3**		**L4**		**L5**	
43							x			
60							x			
63							x			
64							x			
66			x				x	x	x	
91	x	x	x	x	x	x	x	x	x	x
184					x					
186					x		x			
187		x			x	x		x		
194	x	x			x	x	x	x	x	x
199	x	x	x	x	x	x	x	x	x	x
201	x				x		x		x	
207					x				x	
209	x			x		x	x	x		x
227	x		x		x		x	x	x	x

228	x		x		x		x		x	x
229	x		x		x		x		x	
230	x		x		x		x		x	
233	x		x		x		x		x	
234	x		x		x		x		x	
235	x		x		x		x		x	
237				x		x	x	x		x
269			x	x	x			x	x	x
270			x	x	x	x	x	x	x	
288			x							
442						x	x	x		
443			x			x	x	x		x
465			x			x		x		
472			x			x				
481	x	x	x	x	x	x	x	x	x	x
493		x	x	x		x		x		x
494			x	x		x		x		x
497				x		x		x	x	x
499			x	x		x		x	x	x
500				x		x		x	x	x
512				x	x	x	x	x	x	x
513				x		x	x	x	x	x
515	x	x	x	x	x	x	x	x	x	x
516				x		x		x		x
529	x				x		x		x	
531		x	x	x		x		x		

546		x		x	x	x	x	x	x	x
549			x	x	x		x	x	x	
563	x		x	x		x		x	x	x
564	x			x		x		x		x
604					x					
608					x					
609			x		x		x		x	x
614			x							
615	x		x				x			
624	x		x		x					
625	x		x	x	x					
626	x				x					
627	x		x		x		x			
644			x	x	x	x				x
661			x	x		x		x		x
706	x		x		x		x		x	
721	x									x
724	x		x				x	x	x	
768	x		x		x			x		
770	x	x	x	x	x	x	x	x		
786			x	x	x	x	x	x		
856	x		x							
857			x							
868			x							
899					x					
902	x		x	x		x	x	x	x	

905		x		x		x	x	x		x
907					x					
913	x		x							
916	x		x	x	x	x		x		
922									x	
940	x									
945	x			x		x		x		x
947	x					x				
953				x		x		x	x	x
955	x			x		x	x	x	x	x
962	x			x		x				
979	x									
980	x		x		x					
987						x	x			
991	x		x		x	x	x	x		
1007			x					x	x	
1009			x							
1035	x			x		x		x		x
1044	x			x		x		x	x	x
1050			x	x		x				
1059	x		x		x			x	x	
1060		x		x		x		x	x	x
1065	x	x	x	x	x	x	x	x	x	x
1072	x		x						x	
1077	x		x						x	
1082							x			

1085							x			
1087	x	x	x	x	x	x	x	x	x	x
1088							x			
1095			x	x	x	x		x	x	x
1098	x		x	x		x		x	x	x
1106							x			
1120	x	x	x	x	x	x	x	x	x	x
1125							x	x	x	
1137			x							
1138		x		x	x	x	x	x		x
1139	x		x	x	x	x	x	x		x
1141			x							
1142		x	x							
1143		x	x			x				
1152					x					
1165		x	x	x	x	x		x		x
1166				x	x	x				x
1174		x		x	x	x	x	x	x	x
1175									x	
1177		x	x	x						
1184	x		x							
1185	x		x	x	x		x	x		x
1186	x				x					
1191	x		x		x		x		x	
1192			x							
1197	x								x	

1200	x		x		x				x	
1202	x	x	x	x	x		x	x	x	
1204	x		x	x	x	x	x	x	x	x
1207	x		x			x	x	x		x
1218	x								x	
1225					x					x
1253	x		x		x				x	
1255				x	x	x		x	x	x
1257	x		x	x	x	x	x	x	x	x
1261					x					x
1282					x					
1285					x					
1299	x				x		x		x	
1304	x									
1308					x		x		x	
1335	x	x	x	x	x	x		x	x	x
1337			x	x	x	x		x	x	x
1354			x	x		x				
1356	x				x	x				
1366	x	x	x	x	x	x	x	x	x	x
1368				x	x	x		x	x	x
1390					x					
1401			x	x		x		x	x	x
1403	x	x	x	x		x		x		x
1411							x			
1418			x			x		x		

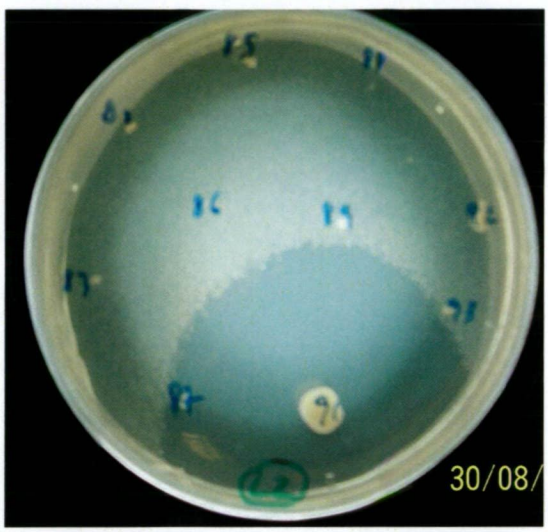
1419	x	x	x	x	x	x	x	x		x
1421			x							
1423	x		x		x		x			
1431		x	x	x			x	x	x	x
1433			x				x		x	
1443	x		x	x		x				
1446				x			x			
1447	x		x	x	x	x	x	x	x	x
1478	x		x	x	x	x	x	x	x	x
1482			x	x	x	x	x	x	x	x
1485			x							
1489		x			x	x		x		
1490					x	x		x		
1493	x									
1496	x	x	x	x	x	x	x	x	x	x
1509			x		x		x		x	
1519				x	x	x	x	x		x
1523			x	x	x	x		x		x
1525	x		x	x	x	x	x	x	x	x
1529			x							
1531	x		x		x					
1532	x		x		x	x	x	x		x
1533	x					x		x		
1536	x		x		x	x		x		
1538	x	x		x		x		x		
1547			x	x	x	x	x	x	x	x

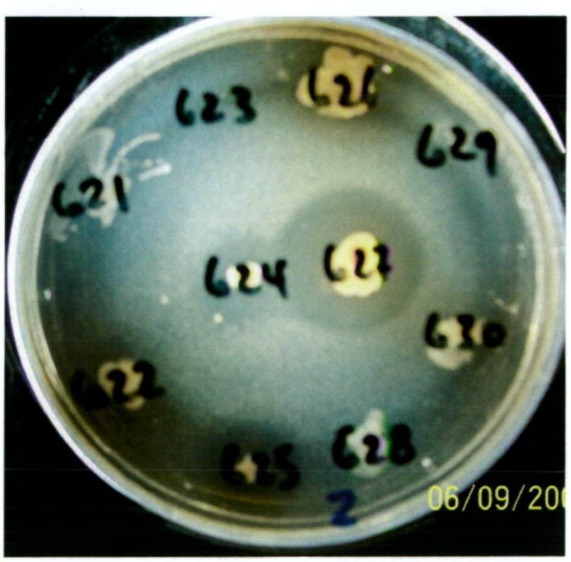
1571	x									
1579							x	x		
1586	x						x	x		
1587	x			x		x		x		
1588	x			x		x	x	x		x
1596				x		x	x	x		x
1599							x			
1606				x	x	x	x	x	x	x
1611	x	x	x	x		x	x	x		
1612	x									
1613	x		x	x	x	x	x	x		x
1615	x		x			x		x		
1616	x	x			x	x	x			
1618	x			x		x				x
1620	x		x		x	x	x	x		
1623	x	x	x		x		x	x		x
1641				x		x	x	x		x
1647	x		x							x
1653			x					x		x
1658		x	x	x		x		x		x
1662			x							
1663			x							
1670	x		x	x		x	x	x		x
1687		x		x		x	x	x		x
1691								x		
1697		x				x		x		x

Actino no.	Zone of inhibition (mm)
91	25
199	15
481	5
515	10
1065	5
1087	5
1120	5
1138	15
1139	5
1174	10
1202	20
1204	5
1257	5
1335	5
1366	15
1419	20
1477	20
1478	5
1496	20
1525	5

Appendix 3: Images of Zones of Clearance - Actinobacteria x *L. monocytogenes* screen

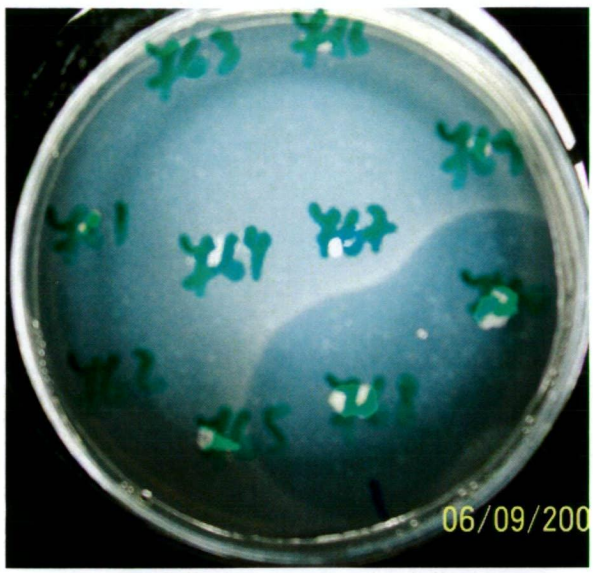
FIRST SCREENING

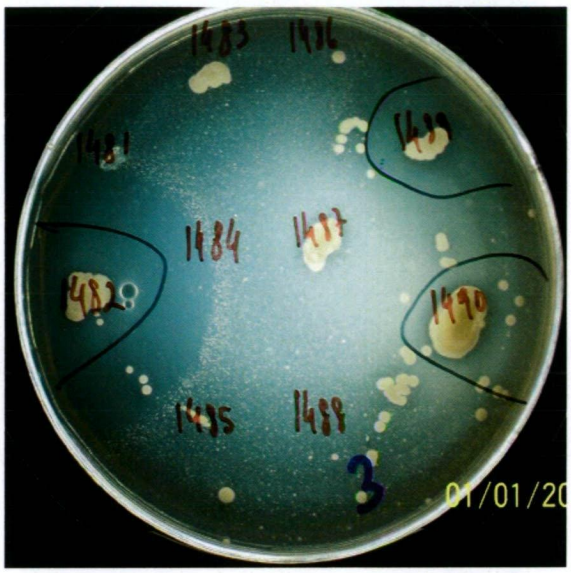




Appendix 3

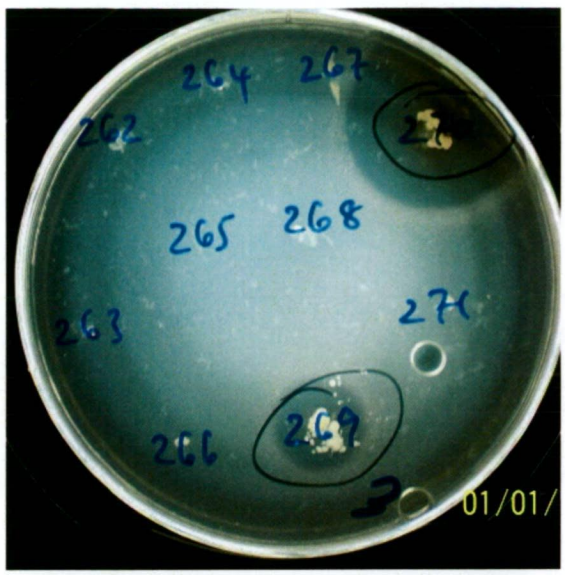
Images of Zones of Clearance





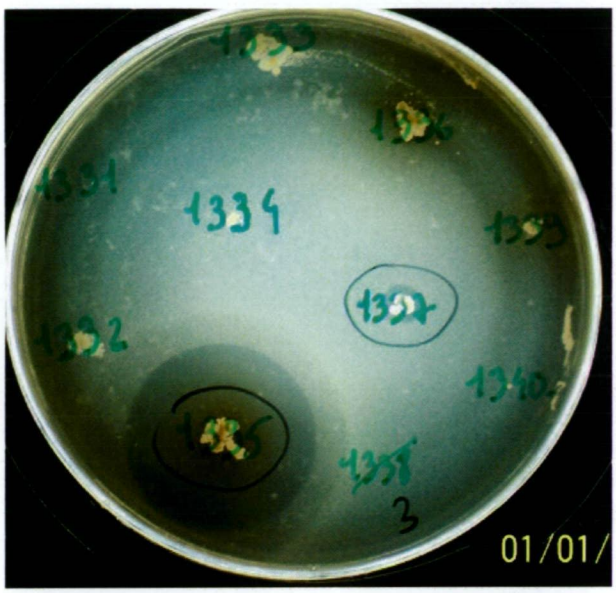
Appendix 3

Images of Zones of Clearance



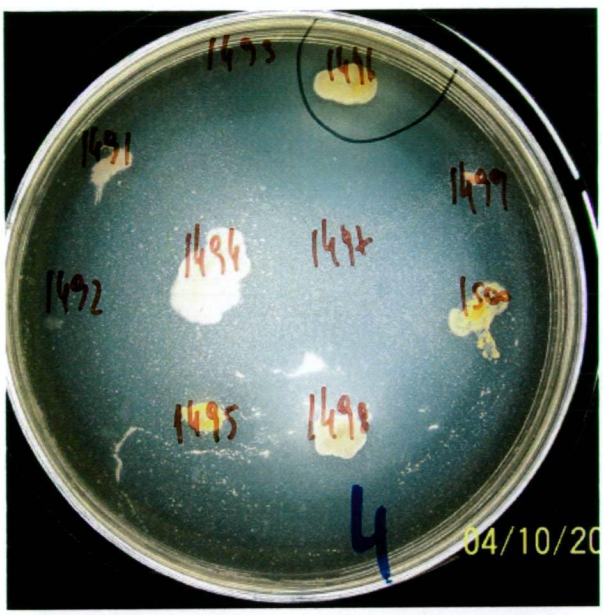
Appendix 3

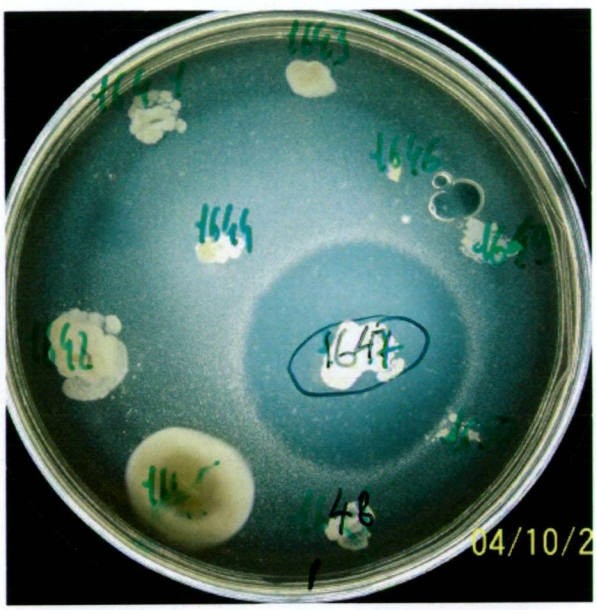
Images of Zones of Clearance

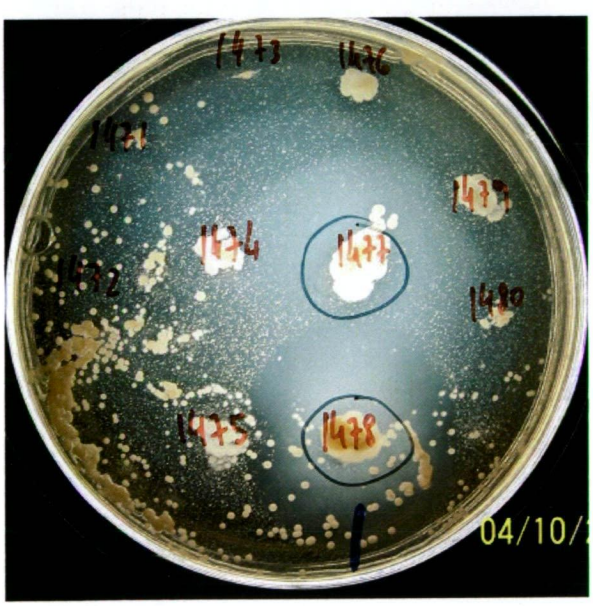


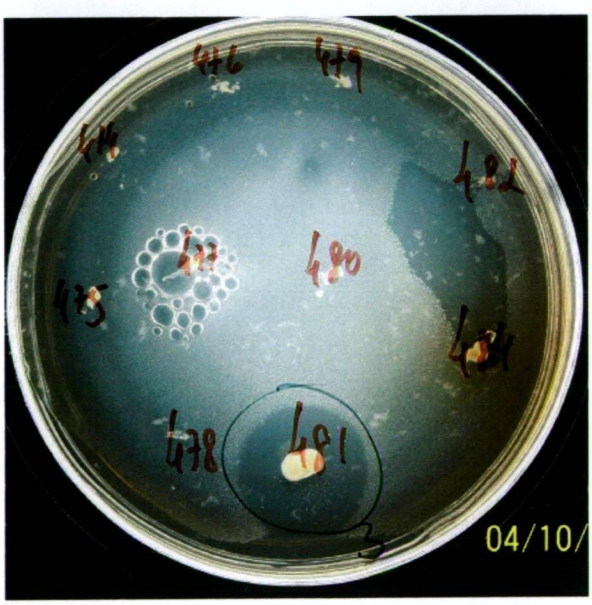
Appendix 3

Images of Zones of Clearance

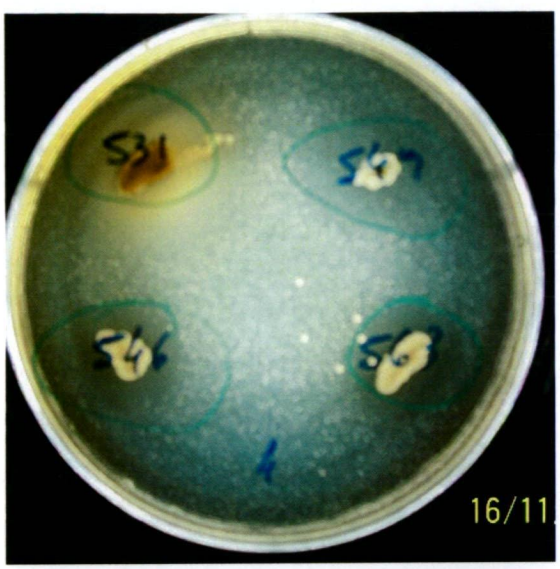


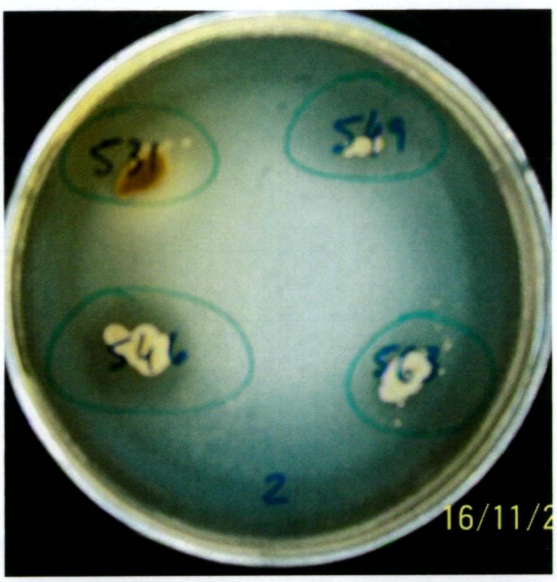


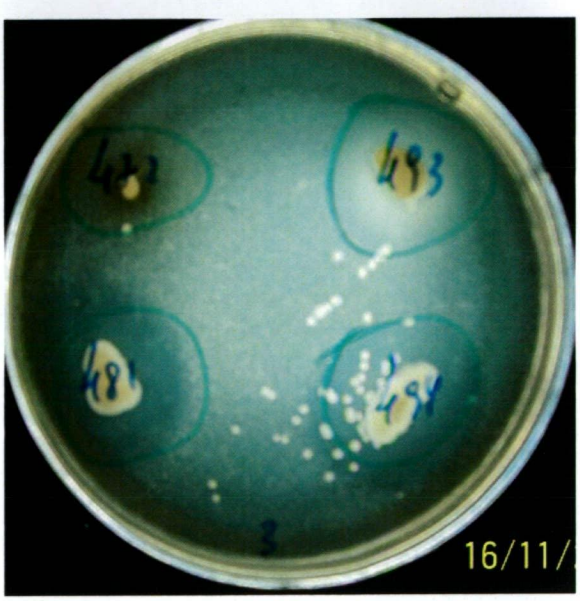




SECOND SCREENING

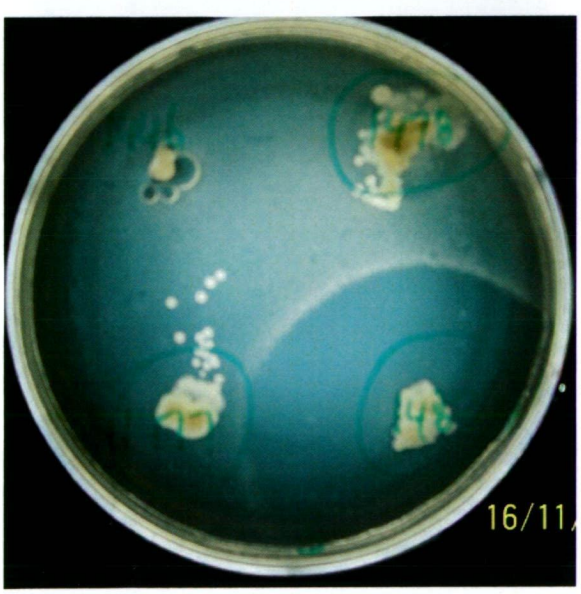


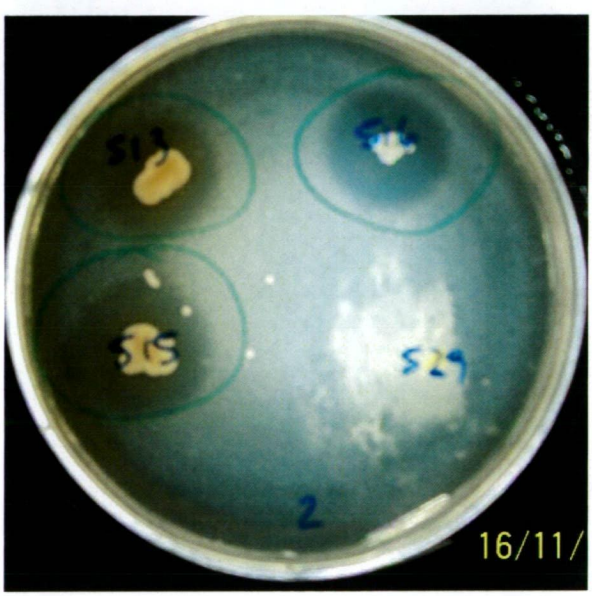




Appendix 3

Images of Zones of Clearance





ENZYMATIC EVALUATION